

UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II



**DOTTORATO DI RICERCA IN
BIOLOGIA, PATOLOGIA E IGIENE AMBIENTALE IN
MEDICINA VETERINARIA**

Indirizzo Morfologia Macroscopica, Microscopica, Ultrastrutturale e
Diagnostica Molecolare

- XXVI CICLO -

**THE COPROLOGICAL DIAGNOSIS OF
GASTROINTESTINAL NEMATODE INFECTIONS
IN SMALL RUMINANTS**

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Anni Accademici 2011-12/ 2013-14

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LIST OF ABBREVIATIONS

AAD: Amino Acetonitrile Derivates

AR: Anthelmintic resistance

BZ: Benzimidazoles

CI: Confidence intervals

CPG: Cysts per gram of faeces

CV: Coefficient of Variation

EPG: Eggs per gram of faeces

FEC: Faecal egg count

FECR: Faecal egg count reduction

FECRT: Faecal egg count reduction test

FS: Flotation solution

FS1: Sheather's sugar solution (Specific gravity = 1.200)

FS2: Saturated sodium chloride (Specific gravity = 1.200)

FS3: Zinc sulphate (Specific gravity = 1.200)

FS4: Sodium nitrate (Specific gravity = 1.200)

FS5: Sucrose and potassium iodomercurate (Specific gravity = 1.250)

FS6: Magnesium sulphate (Specific gravity = 1.280)

FS7: Zinc sulphate (Specific gravity = 1.350)

FS8: Potassium iodomercurate (Specific gravity = 1.440)

FS9: Zinc sulphate and potassium iodomercurate (Specific gravity = 1.450)

GIN: Gastrointestinal nematodes

GI strongyles: Gastrointestinal strongyles

GLM: Generalized linear model

L1: First-stage larvae

L3: Third-stage larvae

LC: Larval culture

LCL: Lower confidence limits

LL: Lower limit

LPG: Larvae per gram of faeces

LSD: Least significant difference

LV: Imidazothiazoles/Tetrahydropyrimidines

McM: McMaster

ML: Macrocyclic lactones

OPG: Oocysts per gram of faeces

PBZ: Probenzimidazoles

PGE: Parasitic gastroenteritis

PP: Periparturient period

PPR: Peri-parturient rise

SD: Standard deviation

RT-PCR: Real-time Polymerase Chain Reaction

SG: Specific gravity

SOP: Standard operating procedures

TST: Targeted selective treatment

TT: Targeted treatment

WAAVP: World Association for the Advancement of Veterinary Parasitology

GENERAL INTRODUCTION

1. THE IMPORTANCE OF GASTROINTESTINAL NEMATODES IN SMALL RUMINANTS

Small ruminant farming has a prominent role in the sustainability of rural communities around the world (Park and Haenlein, 2006), as well as being socially, economically and politically highly significant at national and international levels, as with all livestock species (Morgan et al., 2013). In the European Union (EU), for instance, there are currently around 101 million sheep and 12 million goats (FAOSTAT, 2009). Efficient small ruminant livestock production is also crucial to meet the increasing demands of meat and dairy products, especially in areas in which land is unsuitable for growing crops (Chiotti and Johnston, 1995). Small ruminant dairying is particularly important to the agricultural economy of the Mediterranean region, which produces 66% of the world's sheep milk and 18% of the world's goat milk (Pandya and Ghodke, 2007). However, there are several factors which affect the productivity of the small ruminant livestock sector, the capacity to maintain and improve a farm (i.e. its health and genetic potential) and, as a consequence, also human nutrition, community development and cultural issues related to the use of these livestock (Perry and Randolph, 1999; Nonhebel and Kastner, 2011).

Among the factors that negatively affect the livestock production, infections with parasites and in particular with gastrointestinal nematodes (GIN) continue to represent a serious challenge to the health, welfare, productivity and reproduction of grazing ruminants throughout the world (Morgan et al., 2013; Scala et al., 2011). All grazing animals are exposed to helminth infections at pasture and any respective future intensification of livestock farming will increase the risk of helminth disease. The ranking of GIN as one of the top cause of lost productivity in small and large ruminants by the recent DISCONTTOOLS programme (<http://www.discontools.eu/home/index>) witnesses the increasing EU's consideration of the impact of these parasites upon animal health, welfare and productivity (Vercruysse, 2014). The economic costs of parasitic disease are currently difficult to quantify, however some estimates do exist within the scientific literature; for example, studies in the UK have estimated the cost of GIN of sheep to be in the order of 99m € per year (Nieuwhof & Bishop, 2005). Within

the EU as a whole, annual sales of anthelmintic drugs used to control these infections in ruminants have been estimated to be in the order of 400 million € (Selzer, 2009). It is likely that these figures only represent the tip of the iceberg when it comes to calculating the true cost of livestock helminthoses endemic within the EU (Charlier et al., 2009).

1.1. Gastrointestinal nematodes in small ruminants in Europe – Life cycle and epidemiology

Grazing ruminants are frequently parasitized by multiple species of GIN (Nematoda, Strongylida) which cause the so called parasitic gastroenteritis (PGE) (Kassai, 1999). With respect to small ruminants, GIN parasitizing the abomasum, small and large intestines of sheep and goats include species of *Haemonchus*, *Ostertagia*, *Teladorsagia*, *Trichostrongylus*, *Nematodirus*, *Oesophagostomum*, *Chabertia* and *Bunostomum* (Zajac, 2006) listed in the following Fig. 1.

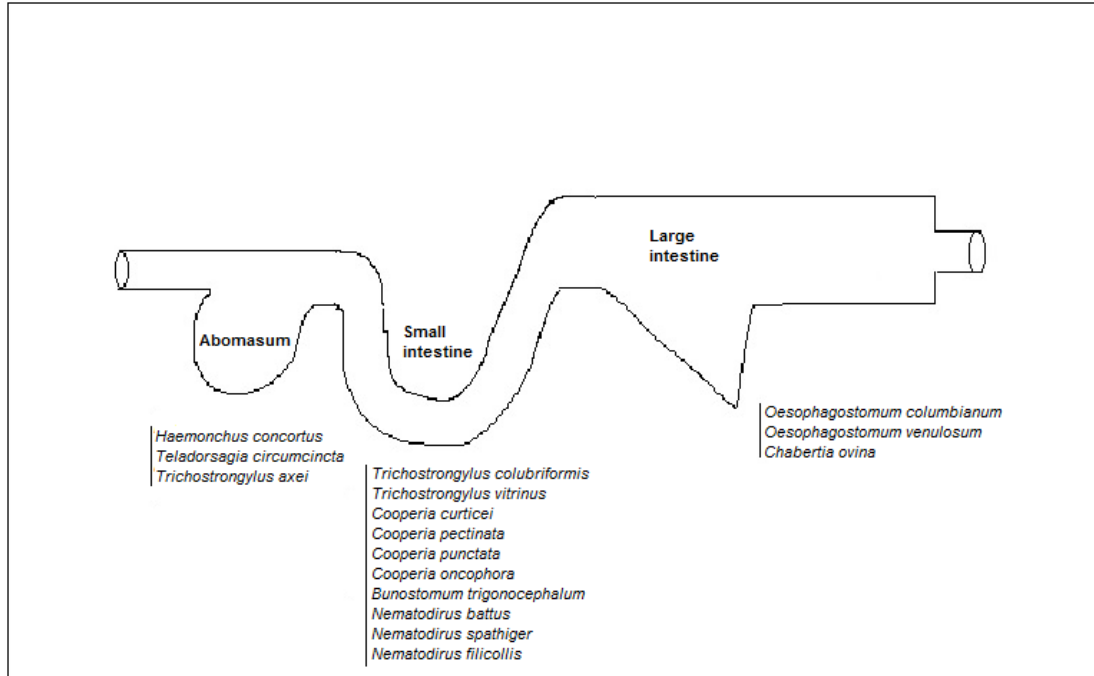


Fig. 1. Location in the host for the prevalent species of GIN infecting small ruminants in Europe.

Some key morphological characteristics (length), pre-patent period, location in the host of the genera of GIN that infect small ruminants in Europe are listed in the following Table 1.

Table 1. The length, pre-patent period, location in the host of the most important genera of GIN infecting sheep in Europe (Anderson, 2000; Taylor et al., 2007; Roeber et al., 2013) .

| Genus | Length (mm) | Pre-patent period (days) | Location in the host |
|-------------------------|--------------------|--------------------------|--------------------------------|
| <i>Haemonchus</i> | ♂ 10-20 ♀ 18–30 | 18-21 | Abomasum |
| <i>Teladorsagia</i> | ♂ 7-8 ♀ 10–12 | 15-21 | Abomasum |
| <i>Trichostrongylus</i> | ♂ 2-8 ♀ 3–9 | 15-23 | Abomasum or small intestine |
| <i>Cooperia</i> | ♂ 4-5 ♀ 5–6 | 14-15 | Small intestine |
| <i>Nematodirus</i> | ♂ 10-19 ♀ 15–29 | 18-20 | Small intestine |
| <i>Bunostomum</i> | ♂ 12-17 ♀ 19–26 | 40-70 | Small intestine |
| <i>Oesophagostomum</i> | ♂ 12-16 ♀ 14–24 | 40-45 | Large intestine |
| <i>Chabertia</i> | ♂ 13-14 ♀ 17–20 | 42-50 | Large intestine |

In general, with some exceptions (e.g. *Nematodirus*, *Bunostomum*), the life cycle of the GIN genera listed in Table 1 follows a similar pattern (Levine, 1968) as shown in Fig. 2. Sexually dimorphic adults are present in the digestive tract, where fertilized females produce large numbers of eggs which are passed in the faeces. Strongylid eggs (70–150 µm) usually hatch within 1–2 days. After hatching, larvae feed on bacteria and undergo two moults to then develop to ensheathed third-stage larvae (L3s) in the environment (i.e. faeces or soil). The sheath (which represents the cuticular layer shed in the transition from the L2 to L3 stage)

protects the L3 stage from environmental conditions but prevents it from feeding. Infection of the host occurs by ingestion of L3s (with the exception of *Nematodirus* for which the infective L3 develops within the egg and of *Bunostomum* for which L3s may penetrate through the skin of the host). During its passage through the stomach, the L3 stage loses its protective sheath and has a histotrophic phase (tissue phase), depending on species, prior to its transition into the L4 and adult stages (Levine, 1968). Under unfavourable conditions, the larvae undergo a period of hypobiosis (arrested development; typical for species of *Haemonchus* and *Teladorsagia*). Hypobiotic larvae usually resume their activity and development in spring in the case of *Haemonchus* or autumn in the case of *Teladorsagia*. This may be synchronous with the start of the lambing season, manifesting itself in a peri-parturient increase in egg production in ewes (Salisbury and Arundel, 1970). The peri-parturient reduction of immunity increases the survival and egg production of existing parasites, increases susceptibility to further infections and contributes to the contamination of pasture with L3s when young, susceptible animals begin grazing (Hungerford, 1990).

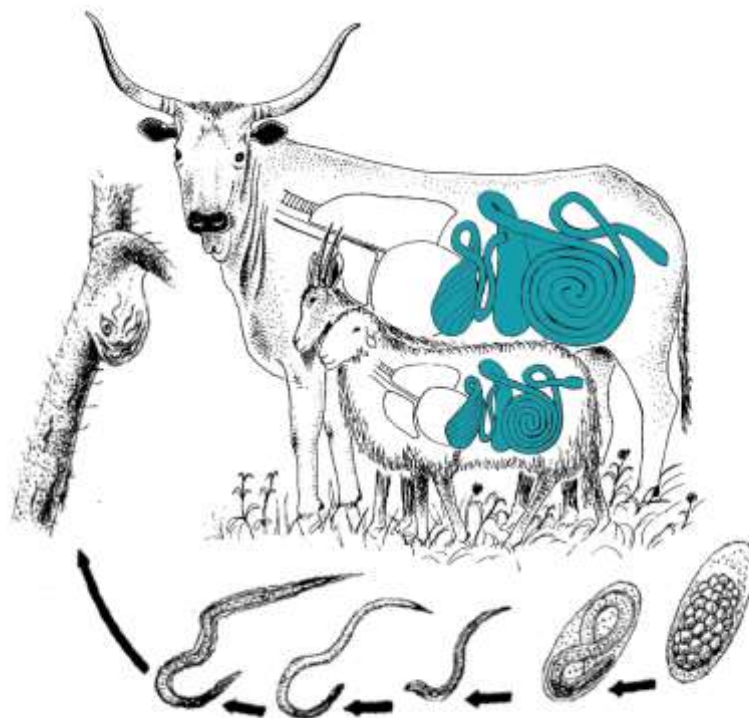


Fig.2. The life-cycle of most species of GIN in ruminants.

The importance of different genera/species of GIN as causes of disease in small ruminants depends not only on their presence, but also their abundance and seasonal patterns of infection. The large number of prevalence surveys and studies of field epidemiology in diverse localities provide a qualitative picture of the distribution and relative importance of different species in Europe. In line with distribution in the southern hemisphere (Kao et al., 2000), *H. contortus* tends to be more common and more of a threat to sheep health and production in warmer, southern areas, while *T. circumcincta* is the dominant nematode of sheep in temperate and northern regions. *Trichostrongylus* and *Nematodirus* spp. are ubiquitous and their importance varies at local scale. *N. battus* is a major cause of disease in lambs only in northern Europe (Morgan and van Dijk, 2012). Update prevalence data on GIN genera in sheep in Europe have been recently generated within the EU-FP7 GLOWORM project. The following Table 2 reports the prevalence data of GIN from 3 key European regions (Ireland, Switzerland, Italy).

Table 2. The prevalence of the most important genera of GIN infecting sheep in Europe (Musella et al. 2011; Dipineto et al. 2013; Gloworm Project - www.gloworm.eu).

| GIN Genera | Italy Prevalence Min-Max (%) | Switzerland Prevalence Min-Max (%) | Ireland Prevalence Min-Max (%) |
|---------------------------------------|------------------------------------|------------------------------------------|--------------------------------------|
| <i>Haemonchus</i> | 56.3 - 72.4 | 71.6 - 81.7 | 3.6 - 6.1 |
| <i>Teladorsagia</i> | 93.8 - 100 | 73.1 - 85.9 | 92.9 - 97.0 |
| <i>Trichostrongylus</i> | 93.8 - 96.6 | 89.5 - 93.9 | 89.3 - 97.0 |
| <i>Cooperia</i> | 12.5 - 34.5 | 28.2 - 32.8 | 33.3 - 60.7 |
| <i>Nematodirus</i> | 35.1 - 53.8 | 33.3 - 38.9 | 61.0 - 68.8 |
| <i>Bunostomum</i> | 0 - 3.4 | 0 - 8.5 | 3.6 - 9.1 |
| <i>Oesophagostomum/ Chabertia</i> | 81.3 - 89.7 | 56.7 - 83.1 | 3.6 - 97.0 |

2. PATHOGENESIS AND PATHOLOGY OF GIN IN SMALL RUMINANTS

Different species of GIN can vary considerably in their pathogenicity, geographical distribution, prevalence and susceptibility to anthelmintics (Dobson et al., 1996). Mixed infections, involving multiple genera and species are common in sheep and

goats, and usually have a greater impact on the host than mono-specific infections (Wimmer et al., 2004). Depending on the number, species and burden of parasitic nematodes, common symptoms of PGE include reduced weight gain or weight loss, anorexia, diarrhoea, reduced production and, in the case of blood-feeding genera (e.g. *Haemonchus*), anaemia and oedema, due to the loss of blood and/or plasma proteins (Kassai, 1999). Usually, low intensities of infection do not cause a serious hazard to the health of ruminants and may be tolerated (i.e. allowing the development of some immunity in the host), but as the numbers of worms increase, subclinical disease can manifest itself and is, therefore, of great economic importance (Fox, 1997; Zajac, 2006). The severity of disease is mainly influenced by factors such as: i) the parasite species - *H. contortus*, *T. circumcincta* and intestinal species of *Trichostrongylus* are considered highly pathogenic in sheep (Besier and Love, 2003); ii) the number of worms present in the gastrointestinal tract; iii) the general health and immunological state of the host; iv) environmental factors, such as climate and pasture type; v) other factors as stress, stocking rate, management and/or diet (Kassai, 1999). Usually, three groups of animals are prone to heavy worm burdens: (i) young, non-immune animals; (ii) adult, immuno-compromised animals; and (iii) animals exposed to a high infection pressure from the environment (Zajac, 2006). Beyond any doubt, a GIN species of primary concern is *H. contortus* (Fig. 3), a highly pathogenic blood-feeder helminth that causes anaemia and reduced productivity and can lead to death in heavily infected animals (Burke et al., 2007).



Fig. 3. An abomasum of a sheep highly infected by *H. contortus*.

3. CONCLUDING REMARKS AND NEEDS FOR RESEARCH

Although representing a significant economic and welfare burden to the global ruminant livestock industry, GIN infections in small ruminants are often neglected and implementation in research, diagnosis and surveillance of these parasites is still poor, mainly in the matter of diagnostic methods and their use/interpretation. The accurate diagnosis (and interpretation) of GIN directly supports parasite control strategies and is of relevance for investigations into parasite biology, ecology and epidemiology (Roeber et al., 2013). This aspect is now particularly important given the problems associated with anthelmintic resistance (AR) in GIN populations of small ruminants worldwide (Roeber et al., 2013 a,b).

Various methods are employed for the ante mortem diagnosis of GIN infections in small ruminants. These include the observation of clinical signs indicative of disease (although non-pathognomonic), coprological diagnosis (faecal egg count – FEC), biochemical and/or serological, and molecular diagnostic approaches (reviewed in Roeber et al., 2013). However, still now, faecal egg count (FEC) techniques remain the most common laboratory methods for the diagnosis of GIN in small ruminants. Also for FEC, widespread standardization of many laboratory techniques does not exist, and most diagnostic, research and teaching facilities apply their own modifications to published protocols (Kassai, 1999). Although these techniques are regarded to be standard diagnostic procedures, there is a lack of detailed studies of diagnostic performance, including the diagnostic sensitivity, specificity and/or repeatability (Roeber et al., 2013). Furthermore, many aspects concerning factors affecting FEC (e.g. season of sampling, sampling period, consistency of faeces, fecundity of worms, etc., as well as interpretation of FEC) have poorly been investigated so far.

These are the reasons that motivated me in choosing “The coprological diagnosis of gastrointestinal nematode infections in small ruminants” as topic of my PhD thesis to help optimize the use and interpretation of FEC in small ruminants.

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CHAPTER 1

Literature review on “The coprological diagnosis of GIN infections in small ruminants”

1. INTRODUCTION

Even in the present era of genomics, metagenomics, proteomics and bioinformatics (Roeber et al., 2013), diagnosis of gastrointestinal nematodes (GIN) in ruminants still relies predominantly on coprological examination (Cringoli et al., 2010; Demeler et al., 2013). Indeed, coproscopy (from the Greek words κόπρος = faeces and -σκοπία = examen), i.e. the analysis of faecal samples for the presence of parasitic elements (e.g. eggs of GIN) is the most widely used diagnostic procedure in veterinary parasitology (Cringoli, 2004). This is the so-called coproscopy *sensu stricto*, instead, coproscopy *sensu lato* is the detection of antigens and/or DNA in faecal samples by immunological (e.g. ELISA) or molecular (e.g. PCR) methods. After foundation of copromicroscopy by C. J. Davaine in 1857, several copromicroscopic techniques (and devices/kits) have been developed, each with its own advantages and limitations. Figure 1 reports a time chart showing the different copromicroscopic techniques (including devices/kits) developed from 1857 to 2013, such as the direct centrifugal flotation method (Lane, 1922), the Stoll dilution technique (Stoll, 1923), the McMaster method (Gordon and Whitlock, 1939), the Wisconsin flotation method (Cox and Todd, 1962) and FLOTAC techniques (Cringoli, 2010).

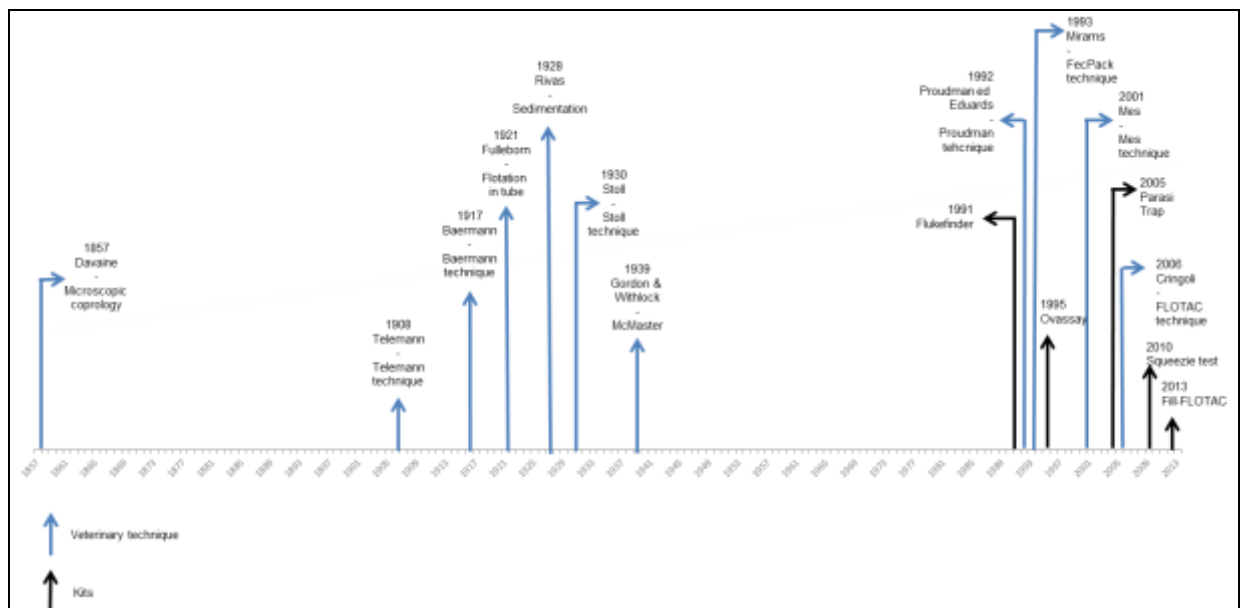


Fig. 1. Time chart showing the different copromicroscopic techniques (including devices/kits) developed from 1857 to 2013.

2. COPROMICROSCOPIC TECHNIQUES

Several manuals of diagnostic veterinary parasitology are available in literature covering multiple animal species, including small ruminants, and describing a plethora of copromicroscopic techniques (MAFF, 1986; Thienpont, 1986; Foreyt, 2001; Hendrix, 2006; Zajac and Conboy, 2012).

Copromicroscopic diagnosis of GIN infections in small ruminants can be either qualitative (thus providing only the presence/absence of GIN eggs) or quantitative, providing also the number of eggs by faecal egg count (FEC). When quantification is pursued (FEC), GIN eggs are counted and usually expressed as the number of eggs per gram (EPG) of faeces.

Qualitative and/or quantitative copromicroscopy in small ruminants usually involves concentration of parasitic elements (e.g. GIN eggs) by either flotation (Fig. 2) or sedimentation (Fig. 3) in order to separate GIN eggs from faecal material.

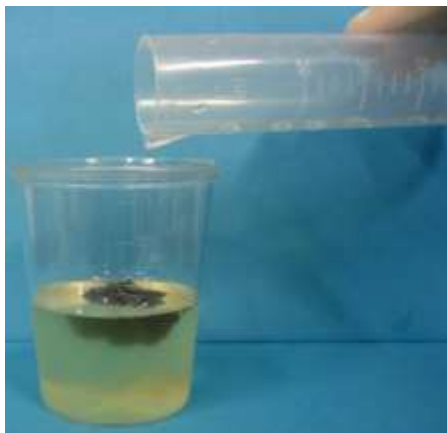


Fig. 2. Flotation technique.



Fig. 3. Sedimentation technique.

The faecal sedimentation concentrates both faeces and eggs at the bottom of a liquid medium, usually tap water. In contrast, the principle of faecal flotation is based on the ability of a flotation solution (FS) to allow less dense material (including parasite eggs) to rise to the top. It should be noted that, in livestock species, sedimentation techniques are considered useless (and time-consuming) to detect GIN eggs, whereas they are very useful for recovering heavy and operculated eggs (e.g. eggs of rumen and liver flukes, Paramphistomidae and

Fasciola hepatica) that do not reliably float or are distorted by the effect of flotation solutions (Dryden et al., 2005). Thus, the methods most frequently used to recover GIN eggs in ruminant faeces are those based on flotation. These procedures are based on differences in the specific gravity (s.g.) of parasite eggs, faecal debris and flotation solution (FS). Most of the FS used in coprology (see Table 1) are saturated and are made by adding a measured amount of salt or sugar (or a combination of them depending on the FS) to a specific amount of water to produce a solution with the desired s.g. After preparing any FS, it is mandatory to check the s.g. with a hydrometer, recognizing that the s.g. of the saturated solution will vary slightly depending on ambient temperature. FS used for copromicroscopic diagnosis of GIN infections in small ruminants are usually based on sodium chloride (NaCl) or sucrose and are characterized by low s.g. (usually 1.200).

Table 1. Flotation solutions (composition and specific gravity) used for copromicroscopy in small ruminants.

| Flotation solution | Composition | s.g.* |
|----------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| Sucrose and formaldehyde | C ₁₂ H ₂₂ O ₁₁ 454 g, CH ₂ O solution (40%) 6 ml, H ₂ O 355 ml | 1.200 |
| Sodium chloride | NaCl 500 g, H ₂ O 1000 ml | 1.200 |
| Zinc sulphate | ZnSO ₄ ·7H ₂ O 330 g, H ₂ O brought to 1000 ml | 1.200 |
| Sodium nitrate | NaNO ₃ 315 g, H ₂ O brought to 1000 ml | 1.200 |
| Magnesium sulphate | MgSO ₄ 350 g, H ₂ O brought to 1000 ml | 1.280 |
| Sodium nitrate | NaNO ₃ 250 g, Na ₂ O ₃ S ₂ ·5 H ₂ O 300 g, H ₂ O brought to 1000 ml | 1.300 |
| Zinc sulphate | ZnSO ₄ ·7H ₂ O 685 g, H ₂ O 685 ml | 1.350 |
| Sodium chloride and zinc chloride | NaCl 210 g, ZnCl ₂ 220 g, H ₂ O brought to 1000 ml | 1.350 |
| Sucrose and sodium nitrate | C ₁₂ H ₂₂ O ₁₁ 540 g, NaNO ₃ 360 g, H ₂ O brought to 1000 ml | 1.350 |
| Sodium nitrate and sodium thiosulphate | NaNO ₃ 300 g, Na ₂ O ₃ S ₂ ·5 H ₂ O 620 g, H ₂ O 530 ml | 1.450 |
| Sucrose and sodium nitrate and sodium thiosulphate | C ₁₂ H ₂₂ O ₁₁ 1200 g, NaNO ₃ 1280 g, Na ₂ O ₃ S ₂ ·5 H ₂ O 1800 g, H ₂ O 720 ml | 1.450 |

*Specific gravity

Copromicroscopic diagnosis of GIN in small ruminants is usually performed by quantitative techniques. All FEC techniques which assess the number of helminth eggs per gram of faeces (EPG) and use flotation are based on the microscopic examination of an aliquot of faecal suspension from a known volume of a faecal sample (Nicholls and Obendorf, 1994).

FEC in small ruminants and other livestock species can be performed using different techniques/devices as McMaster (Fig. 4), FecPak (Fig. 5), the flotation in centrifuge (Cornell-Wisconsin technique) (Egwand and Slocombe, 1982) (Fig. 6), or using specific devices as FLOTAC and its derivatives Mini-FLOTAC and Fill-FLOTAC (Fig. 7).

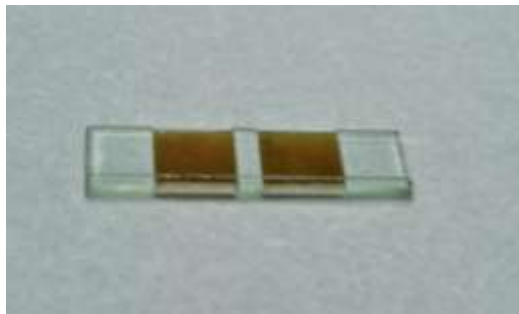


Fig. 4. McMaster

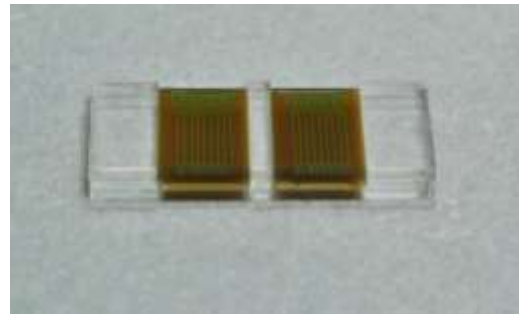


Fig. 5. FECPAK



Fig. 6. Flotation in centrifuge (Cornell-Wisconsin technique).



Fig. 7. Devices of the "FLOTAC Family": Mini-FLOTAC, FLOTAC and Fill-FLOTAC.

The McMaster technique developed and improved at the McMaster laboratory of the University of Sidney (Gordon and Whitlock, 1939; Whitlock, 1948), and whose name derives from one of the great benefactors in veterinary research in Australia, the McMaster family (Gordon, 1980), is the most universally used technique for estimating the number of helminth eggs in faeces (Rossanigo and Gruner, 1991;

Nicholls and Obendorf, 1994). For decades, numerous modifications of this method have been described (Levine et al., 1960; Raynaud, 1970; Roberts and O'Sullivan, 1950; Whitlock, 1948), and most teaching and research institutions apply their own modifications to existing protocols (Kassai, 1999). Many of these modifications make use of different FS, sample dilutions and counting procedures, which achieve varying analytic sensitivities (Cringoli et al., 2004; Roeber et al., 2013). There are at least three variants of the McMaster (for details see MAFF, 1986) with different analytic sensitivities: 50 EPG for the 'modified McMaster method' and the 'modified and further improved McMaster method' or 10 EPG in the case of the 'special modification of the McMaster method.

FECPAK is a derivative of McMaster, developed in New Zealand to provide a simple "on farm" method of egg counting for making decisions on the need to treat or to determine whether anthelmintics are effective. It is essentially a larger version of the McMaster slide (www.fecpak.com), having a high analytic sensitivity (usually 10-30 EPG). The use of such a system requires a significant level of cooperation by farmers and adequate training to ensure that correct diagnoses are made (McCoy et al., 2005).

FEC methods that involve flotation in centrifuge include Cornell-Wisconsin (Egwand and Slocombe, 1982) and FLOTAC (Cringoli et al., 2010) both allowing for the detection of GIN up to 1 EPG.

Cornell-Wisconsin (analytic sensitivity = 1 EPG) is based on flotation in a centrifuge tube and eggs are recovered by means of adding a cover slide to the meniscus of the flotation solution; when the number of eggs is high, inefficiencies may arise due to the lack of precision in the egg counting procedures owing to the absence of a grid on the coverslip.

The FLOTAC techniques are based on the centrifugal flotation of a faecal sample suspension and subsequent translation of the apical portion of the floating suspension. FLOTAC device can be used with three techniques (basic, dual and double), which are variants of a single technique but with different applications. The FLOTAC basic technique (analytic sensitivity = 1 EPG) uses a single FS and the reference units are the two flotation chambers (total volume 10 ml, corresponding to 1 g of faeces). The FLOTAC dual technique (analytic sensitivity = 2 EPG) is based on the use of two different FS that have complementary specific gravities (s.g.) and

are used in parallel on the same faecal sample. With the FLOTAC dual technique, the reference unit is the single flotation chamber (volume 5 ml; corresponding to 0.5 g of faeces). The FLOTAC double technique (analytic sensitivity = 2 EPG) is based on the simultaneous examination of two different faecal samples from two different hosts using a single FLOTAC apparatus. With this technique, the two faecal samples are each assigned to its own single flotation chamber, using the same FS. With the FLOTAC double technique, the reference unit is the single flotation chamber (volume 5 ml; corresponding to 0.5 g of faeces).

A main limitation of FLOTAC is considered the complexity of the technique which involve centrifugation of the sample with a specific device, equipment that is often not available in all laboratories. To overcome these limitations, under the “FLOTAC strategy” of improving the quality of copromicroscopic diagnosis, a new simplified tool has been developed, i.e. the Mini-FLOTAC, having an analytic sensitivity of 5 EPG (Cringoli et al., 2013). It is a easy-to-use and low cost method, which does not require any expensive equipment or energy source, so to be comfortably used to perform FEC (Cringoli et al., 2013). It is recommended that Mini-FLOTAC be used in combination with Fill-FLOTAC, a disposable sampling kit, which consists of a container, a collector (2 or 5 gr of faeces) and a filter. Hence, Fill-FLOTAC facilitates the performance of the first four consecutive steps of the Mini-FLOTAC technique, i.e. sample collection and weighing, homogenisation, filtration and filling (Fig. 8).

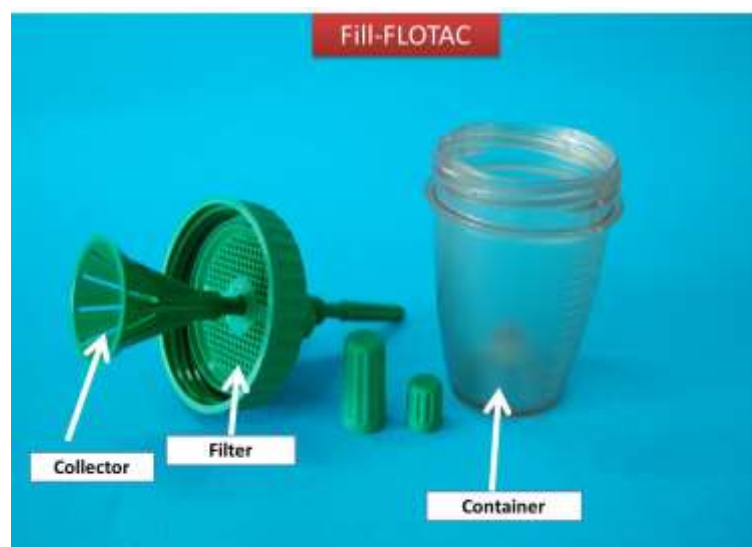


Fig 8. The main components of Fill-FLOTAC.

The Appendix of this chapter reports the standard operating procedures (SOP) of the FEC techniques mostly used for the diagnosis of GIN in small ruminants, namely McMaster, Wisconsin, FLOTAC and Mini-FLOTAC. The following scheme (Fig. 9) shows the main characteristics (volume and reading area) and analytic sensitivities (multiplication factors when a dilution ratio of 1:10 is used) of the FEC techniques mostly used for the diagnosis of GIN in small ruminants.












| FEC Techniques | Volume (ml) | Reading Area (mm ²) | Analytic Sensitivity (Multiplication Factor) (Dilution ratio 1:10) |
|-------------------------------------------------------------------------------------|-------------|---------------------------------|--------------------------------------------------------------------|
| MCMMASTER | | | |
|  | 0.15 ml | 100 mm ² | 66.6 |
|  | 0.30 ml | 200 mm ² | 33.3 |
|  | 0.50 ml | 324 mm ² | 20 |
|  | 1.0 ml | 648 mm ² | 10 |
| FECPAK | | | |
|  | 0.5 ml | 216 mm ² | 20 |
|  | 1.0 ml | 432 mm ² | 10 |
|  | 1.4 ml | 546 mm ² | 7.1 |
|  | 2.8 ml | 1092 mm ² | 3.6 |
| CORNELL-WISCONSIN | | | |
|  | 15 ml | 324 mm ² | 1 |
| FLOTAC | | | |
|  | 10 ml | 648 mm ² | 1 |
| Mini-FLOTAC | | | |
|  | 2 ml | 648 mm ² | 5 |

Fig. 9. Schematic features (volume, reading area, analytic sensitivity at 1:10 dilution ratio) of McMaster, FecPak, Cornell-Wisconsin, FLOTAC and Mini-FLOTAC techniques.

It should be noted that each of the FEC technique described above shows strengths and limitations. Furthermore, they vary considerably according to their performance and operational characteristics (e.g. analytic sensitivity, accuracy and precision in assessing FEC, timing and ease of use).

3. IDENTIFICATION OF GIN EGGS AND COPROCULTURES

For most GIN genera/species there is an overlap in size of the eggs (Fig. 9 a,b,c); only *Nematodirus* (Fig. 9 d) is an exception because its eggs are sufficiently different for their differentiation by size and shape (Table 2).

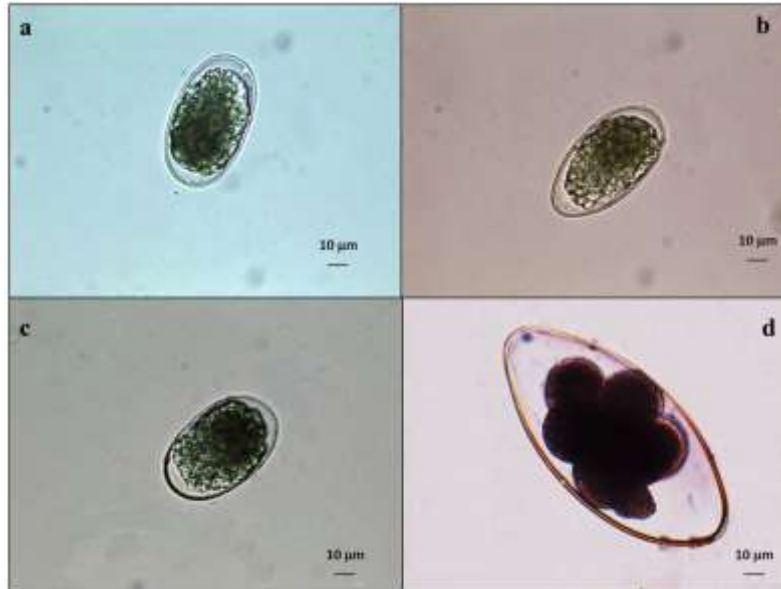


Fig. 9. GIN eggs (a,b,c) and *Nematodirus* egg (d) .

Table 2. Morphometric characteristics of the eggs of different genera of GIN infecting small ruminant: size (µm), shape and shell (data from Thienpont, 1986).

| Genus | Size (µm) | Shape | Shell |
|-------------------------|------------------|-------------------------------------------------------------------------------------|-------|
| <i>Haemonchus</i> | 62-95 X 36-50 | Oval; the eggs contain numerous blastomeres hard to distinguish | Thin |
| <i>Teladorsagia</i> | 74-105 X 38-60 | Oval; the eggs contain numerous blastomeres hard to distinguish | Thin |
| <i>Trichostrongylus</i> | 70-125 X 30-55 | Oval; the eggs contain 16 to 32 blastomeres | Thin |
| <i>Cooperia</i> | 60-95 X 29-44 | Oval with parallel sides; the eggs contain numerous blastomeres hard to distinguish | Thin |
| <i>Nematodirus</i> | 152-260 X 67-120 | Oval; the eggs contain numerous blastomeres hard to distinguish | Thin |
| <i>Bunostomum</i> | 75-104 X 45-57 | Oval; the eggs contain 4 to 8 blastomeres | Thin |
| <i>Oesophagostomum</i> | 65-120 X 40-60 | Oval; the eggs contain 16 to 32 blastomeres | Thin |
| <i>Chabertia</i> | 77-105 X 45-59 | Oval; the eggs contain 16 to 32 blastomeres | Thin |

Therefore, to aid the identification of different GIN present in mixed infections, FEC has to be followed by faecal culture to identify infective third-stage larvae (L3) of GIN. Currently, a number of protocols for coprocultures have been published which differ in the temperatures, times and media used for culture and the approach of larval recovery (reviewed in Roeber et al., 2013). The most widely employed protocol suggests incubation of faeces at 27 °C for 7 days (MAFF, 1986). However, studies have shown that different species of GIN require different conditions, such as environmental temperature and relative humidity, to enable adequate development (Beveridge et al., 1989; O'Connor et al., 2006). This is particularly important to consider when larval culture (LC) results are used to estimate the contribution of different species to mixed infections. One culture protocol is likely to favour the development of one species over others (Dobson et al., 1992). For instance, Whitlock (1956) observed that culture conditions (27 °C for 7 days) are suitable for most species, but that the free-living stages of *Teladorsagia* species develop better at somewhat lower temperatures. Similarly, Dobson et al. (1992) demonstrated that the developmental success of L3 in faecal cultures was lower for *Te. circumcincta* than for *T. colubriformis* when cultured alone or concurrently, thus indicating that LCs are unreliable for estimating the contribution of individual species in mixed infections. In another study, Berrie et al. (1988) also concluded that faecal culture and subsequent larval differentiation are unsuitable for an accurate estimation of the proportions of individual species in mixed infections and can only be used to provide an indication of the species present.

Further variability in the results obtained from LCs have been attributed to differences in the composition of the culture medium used, which influences the moisture, oxygen availability and/or pH that larvae encounter during their development (Hubert and Kerboeuf, 1984; Roberts and O'Sullivan, 1950). Hubert and Kerboeuf (1984) developed a modified method of LC using an 'on-agar' approach to provide standardized conditions. Their results showed that the culture on agar medium led to higher recoveries of larvae compared with traditional faecal cultures. However, lengthy preparation times and increased laboratory requirements appear to limit the routine application of this method.

In addition to the variability of results related to the culture conditions employed, the specific identification of cultured larvae provides challenges (Roeber et al., 2013). For the identification of infective L3s (Fig. 10) to the species or genus level (Table 3), a number of different approaches have been described. A commonly employed method involves the detection of particular morphological features of the larvae (e.g. the length of the tail sheath extension and total body length of L3s) and their comparison with published identification keys (Dikmans and Andrews, 1933; Gordon, 1933; MAFF, 1986; McMurtry et al., 2000; van Wyk et al., 2004). Various keys for the identification of L3s have been published (Dikmans and Andrews, 1933; Gordon, 1933; MAFF, 1986) and a substantial variability in the length of L3s has been reported by different authors (McMurtry et al., 2000). Van Wyk et al. (2004) developed a simplified approach which uses the mean length of the tail sheath extension (Table 3) to differentiate L3s of *Teladorsagia* and/or *Trichostrongylus* from the larvae of *Haemonchus* and *Chabertia* and/or *Oesophagostomum*. However, this approach has the disadvantage that it does not allow the unequivocal differentiation of all genera. For instance, *Teladorsagia* and *Trichostrongylus* cannot be differentiated based on sheath extension length alone. To further refine their differentiation, additional morphological features are required. Lancaster and Hong (1987) proposed the presence of an inflexion at the cranial extremity of *Teladorsagia* larvae as an informative morphological feature. However, this feature is very subtle and its detection is subjective. Another approach to differentiate L3s of *Teladorsagia* from those of *Trichostrongylus* was proposed by Gordon (1933); it is based on the body length measurements of the larvae. The differentiation of L3s of *Oesophagostomum* and *Chabertia* is not considered possible using current techniques for larval differentiation.

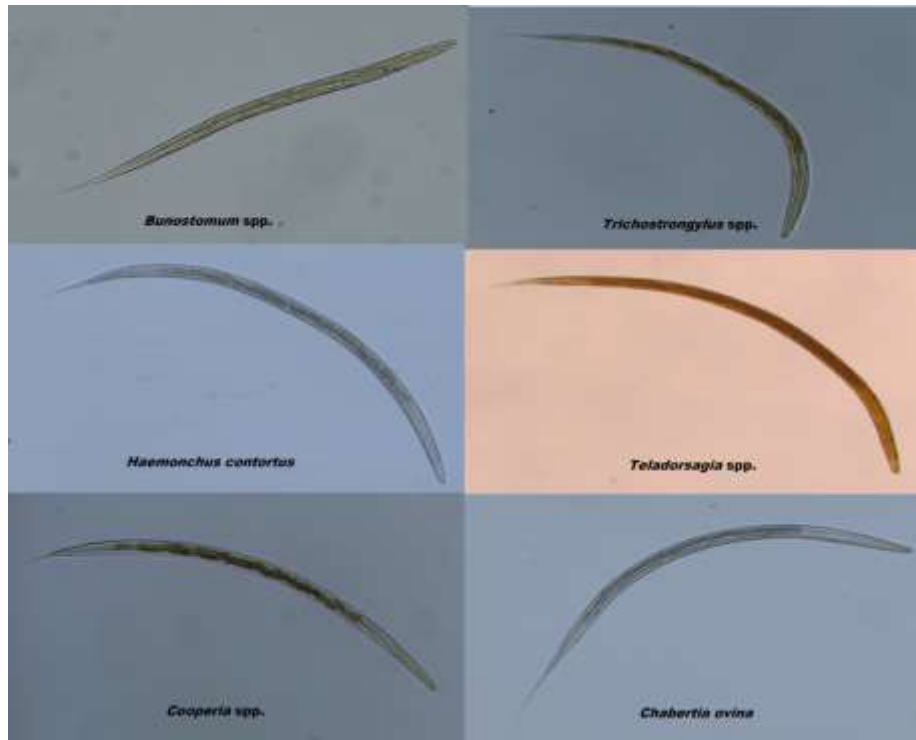


Fig. 10. Third-stage larvae (L3s) of key species of GIN of sheep, encountered following larval culture.

Table 3. Total length and length of the sheath tail of L3 of GIN infecting small ruminants (from van Wyk et al. 2004).

| Genus | Total length (μm) | sheath tail (μm) |
|-------------------------|--------------------------------|-------------------------------|
| <i>Haemonchus</i> | 604 - 720 | 65-82 |
| <i>Teladorsagia</i> | 691 - 806 | 30-44 |
| <i>Trichostrongylus</i> | 590 - 691 | 18-31 |
| <i>Cooperia</i> | 666 - 956 | 62-82 |
| <i>Nematodirus</i> | 752 -1248 | 267-309 |
| <i>Bunostomum</i> | 560 - 633 | 85-115 |
| <i>Oesophagostomum</i> | 720 - 864 | 122-207 |
| <i>Chabertia</i> | 734 - 792 | 101- 150 |
| | | |

A less commonly used method for larval differentiation involves the culture and morphological identification of L1s (Whitlock, 1959). This technique has the advantage of being rapid, since the time required for the development of the L1 stage is shorter; however, the same limitations for the culture and identification of L3s exist for L1s and L2s (Lichtenfels et al., 1997).

For the reasons described above, some recent developments have been made towards improving species identification and differentiation of GIN. These include lectin staining for the identification of *H. contortus* eggs (Palmer and McCombe, 1996), computerized image recognition of strongylid eggs (Sommer, 1996), as well as immunological and molecular methods (Roeber et al., 2013).

PCR-based methods using specific genetic markers in the internal transcribed spacers of nuclear rDNA are considered enhanced tools to differentiate GINs. For instance, recent studies have demonstrated that real-time PCR (RT-PCR) and multiplexed-tandem PCR (MT-PCR) assays can replace the method of larval culture (Roeber et al., 2011, 2013). This test improves the diagnosis of infections with nematode species, which are problematic to detect or identify by traditional coprological techniques, either because of their morphological/morphometric similarity with other species/genera (i.e., *Teladorsagia* and *Trichostrongylus*, *C. ovina* and *O. venulosum*) or their unfavourable development under 'standard' culture conditions (Roeber et al., 2013). In the next future the use of high-throughput immunological and molecular-based technologies will offer the potential for multiplex, high-throughput diagnosis of GIN. As an example, the advent of microbead-based technologies has led to the development of a number of multiplex assay platforms e.g. LUMINEX®, that will permit multiple assays to be performed on the same samples and provide a range of versatile assay designs, including antibody/antigen, primer/probe and enzyme/substrate interactions, also for GIN (www.gloworm.eu).

4. FACTORS AFFECTING FEC OF GIN IN SMALL RUMINANTS

Interpretation of FEC results is of primary importance towards monitoring and controlling GIN infections in small ruminants. The previous section provided an overview of the different FEC techniques available for the diagnosis of GIN in small ruminants. From a general point of view, the method of copromicroscopy to be chosen should depend on what the information is going to be used for. Veterinarians, parasitologists and their staff should re-evaluate their attitude of "it's only a faecal sample" and should therefore consider that suitable and timely

sampling is the pre-requisite for interpreting the results of FEC in order to: estimate infection intensity (McKenna, 1987; McKenna and Simpson, 1987), determine the degree of contamination with helminth eggs (Gordon, 1967), assess the effectiveness of anthelmintics (Waller et al., 1989), determine the breeding value of an animal when selecting for worm resistance (Woolaston, 1992), and guide control and treatment decisions (Brightling, 1988).

FEC results will depend on a plethora of different factors which include: storage of faecal sample, consistency (water content) of faeces, but also biological/epidemiological factors related either to the parasite or to the host (e.g. fecundity of worms, season of sampling, age and sex of animals, immunity development, etc).

Storage conditions of faecal samples are of importance because, if not performed appropriately, they can cause a significant reduction in GIN egg numbers. An artefactual reduction in FECs occurs primarily due to hatching of eggs or biological degradation (Nielsen et al., 2010). To circumvent this problem, different strategies, such as chemical preservation (Whitlock, 1943) or refrigeration (Nielsen et al., 2010) have been recommended but the aspects concerning storage conditions deserve further investigation. It is important to underline that faecal samples should be put into individual labelled containers/gloves and sent promptly for FEC. If nematode larvae are to be cultured for identification, samples should not be stored at 4-8°C for more than 24 h as this may affect the hatching of eggs of *H. contortus* and *Cooperia* (McKenna, 1998).

Consistency (water content) of the faecal sample is another aspect of great relevance for FEC interpretation. Indeed, samples intended for faecal analysis can be of varying consistencies, being soft to watery (diarrhoeic) or hard and desiccated (mostly from animals following transport and without access to food or water) (Gordon, 1953, 1981). These aspects are of importance, as the water content of the sample can either dilute or concentrate the numbers of GIN eggs determined from 1 g of faeces (EPG), depending on the actual amount of dry matter (Le Jambre et al., 2007).

Fecundity of the different species of GIN is also another important factor affecting FEC. The biotic potential of different species of GIN varies (Gordon, 1981) and parasite density and immune mediated 'control' by the host have been shown to

influence the egg production of female worms in different species (Rowe et al., 2008; Stear and Bishop, 1999). Indeed, some GIN as *H. contortus* are known to be highly fecund species (Robert and Swan, 1981), whereas some others show a low fecundity, such as species of *Teladorsagia (Ostertagia)* (Martin et al., 1985), *Trichostrongylus* (Sangster et al., 1979) and *Nematodirus* (Martin et al., 1985; McKenna, 1981).

Also, the seasonal patterns of GIN infection in small ruminants should be considered as factor affecting FEC, in order to select the best period (months) of conducting helminth egg counts. A good knowledge of GIN epidemiology in a given area could be of great interest when deciding the best period to conduct a FEC in small ruminants. GIN egg counts are strongly influenced by the period of sampling (seasonality) and will vary greatly from one month to the next, one year to the next and between geographical locations depending on the prevailing climatic conditions (Cringoli et al., 2008; Morgan et al., 2013). The following Figure 11 shows a typical seasonal pattern of GIN egg counts in sheep in southern Italy (a region with a Mediterranean climate) with two peaks of EPG (February and November) and a ditch (May to June).

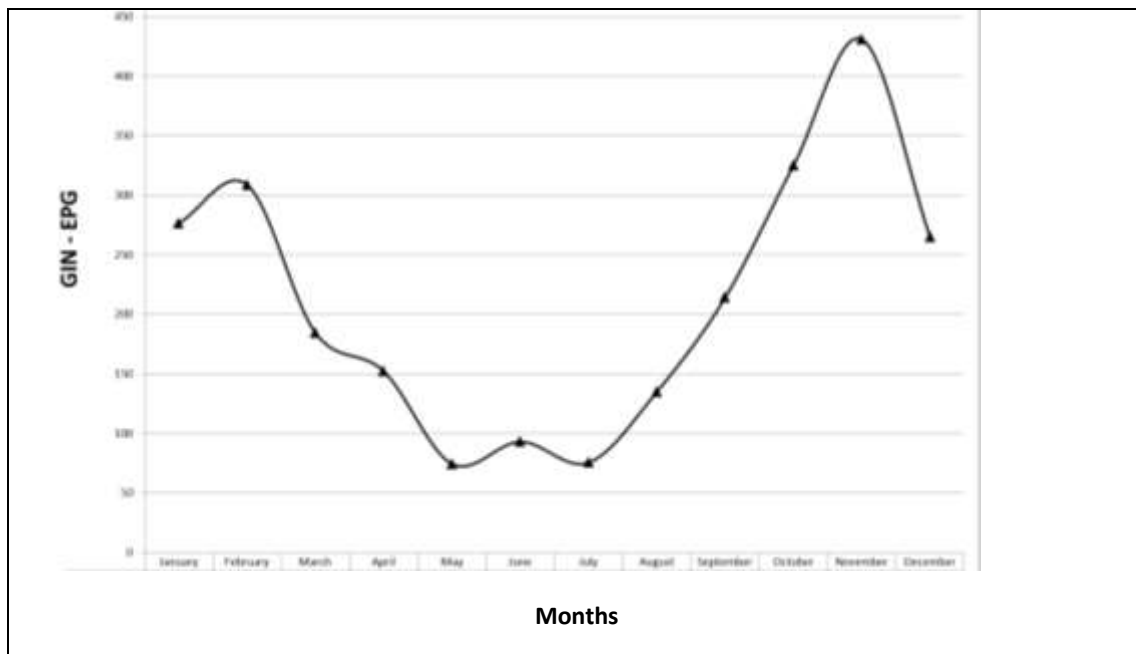


Fig.11. GIN egg count pattern in sheep in southern Italy.

Similarly, Doligalska et al. (1996) showed that FEC variation is usually continuous but heavily skewed in sheep in Poland where the mean and variance of FEC differ

within seasons and years of sampling (Doligalska et al., 1996). Other studies performed in Canada, demonstrated that GIN peaks occur in spring for the ewes and in summer for the lambs (Mederos et al., 2010). McMahon et al. (2012), in studies performed in Northern Ireland, showed that pasture contamination levels of GIN are at their highest over the period September-October having increased steadily over the immediately preceding months (March–May) (McMahon et al., 2012).

Other important factors affecting FEC in small ruminants include the physiological status of the animals. It is well known that high GIN egg production is usually observed in ewes during the periparturient period (PP). The so called periparturient rise (PPR) is a major source of GIN pasture contamination for both lambs and ewes (Barger, 1999; Scala et al. 2012). Dunsmore (1965) suggested that both environmental and physiological factors might be important contributors to the PPR. Some authors believe the PPR is linked to the ewes' productivity stage, and the endocrine, immunological, and metabolic changes that ensue (Taylor, 1935; Crofton, 1954; Brunsdon, 1970; Michel, 1976; Jeffcoate and Holmes, 1990; Coop and Holmes, 1996; Donaldson et al., 1998; Beasley et al., 2010b). Beasley et al. (2010b) showed that changes consistent with a reduction in immunity expression occurred in both pregnant and lactating ewes. These changes in immunity may facilitate the parasites' establishment within the host, enhance their prolificacy, and increase their longevity (Michel, 1976).

Another problem is the effect of the development of host immunity on rate of egg laying by GIN. The first effect on worms of developing host immunity is a reduction in egg laying so there is then no relationship between numbers of worms and egg counts. So whilst FEC may give an indication of worm burdens in young animals this no longer applies in older animals, unless the host species develops little or no natural immunity. Additional considerations are that FECs (i) only reflect patent but not pre-patent infections (Thienpont et al., 1986), (ii) do not provide any information regarding male or immature worms present (McKenna, 1981) and (iii) can be influenced by variation in times of egg excretion by adult worms (Villanua et al., 2006), age of the worm population and/or the immunity, age and sex of the host (Thienpont et al., 1986).

All the findings described above underline that several host-parasite-related

factors could strongly affect FEC and therefore these factors should be taken into consideration when interpreting FEC results. Therefore, FEC alone should not be used to make a diagnosis or guide treatment decisions, but should be interpreted in conjunction with information about the nutritional status, age and management of sheep/goats in a flock (McKenna, 2002).

5. THE USE (INTERPRETATION) OF GIN EGG COUNTS IN SMALL RUMINANTS

The use of FEC in small ruminants and other livestock species has several important purposes. The first is to determine whether animals are infected and to estimate the intensity of infection. The second is to determine whether animals need to be treated to improve their health with the resulting increase of productive performance. The third is to predict pasture contamination by parasitic eggs. The fourth is to determine the efficacy of anthelmintics (FEC reduction – FECR) as well as monitoring control programs.

However, as described also in the previous section, the problem with interpreting FEC is of great relevance. A first issue to consider is that, at least in grazing animals, infections with GIN are usually multi species. Thus, with different species of worms laying eggs at different rates only estimates can be given for determining the intensity of infection and therefore deciding when animals should be treated. Some GIN species permit the natural development of immunity so that using FEC to decide whether treatment is required is a balance between permitting development of immunity and avoiding loss of productivity.

A second important point to consider is the relation between FEC and worm burden. Indeed, there is a controversial debate in the literature to establish whether FEC results may predict the intensity of GIN infection. The relation between FEC and worm burden is a multifactorial issue and will depend on: i) the FEC technique employed; ii) the host and the parasite species involved. For example, FEC results for adult cattle are of limited diagnostic value for determining intensity of infection, as they do not usually correlate with worm burden (McKenna, 1981). Furthermore, FECs in cattle are usually low and require more sensitive flotation techniques than for sheep (Mes et al., 2001); for species of *Nematodirus*, egg counts are also regarded to be of limited value, as most damage is

caused by the immature stages before egg-laying commences (McKenna, 1981). In small ruminants infected with *H. contortus* (Roberts and Swan, 1981; Coadwell and Ward, 1982) or *T. colubriformis* (Beriajaya and Copeman, 2006) FEC is strongly correlated with worm burden. However, this relationship does not hold true for infection with other nematode species, especially *Nematodirus* spp. (Coles et al., 1986) and *T. circumcincta* (Jackson and Christie, 1979). In addition, in areas where co-infection with many nematode species occurs, the high relatively high egg production of *H. contortus* may tend to mask the much lower egg production of species such as *T. colubriformis* and *T. circumcincta* (Roeber et al., 2013). Overall, exploring the relationship between FEC and worm burden is of primary importance in small ruminant practice and needs further investigation. The use of FEC is considered important to indicate levels of pasture contamination, triggering group treatment in the interests of longer-term reduction in infection pressure by GIN, in concert with pasture movement and rotation schemes (Kenyon et al., 2014). FEC has long been used in sheep and goat production systems, to focus group anthelmintic treatments for example at times of high challenge in growing lambs, or high egg production in peri-parturient ewes.

With respect to sheep, as already mentioned in the previous sections, the number of GIN eggs in a faecal sample varies with factors related to the host and parasite species. This aspect should be taken into account to identify FEC thresholds for treatment. Indeed, not only there are no widely accepted defined FEC thresholds for treatment, but also these thresholds will vary between the different nematode species (Kenyon et al., 2014). Some authors suggest that less than 500 EPG is considered a low level of GIN infection, between 500 and 1500 EPG as moderate to high, and more than 1500 EPG as high level of infection (Hansen and Perry, 2000). According to other authors FEC of ≥ 200 EPG is regarded to indicate a significant worm burden and is used as basis for the decision for anthelmintic treatment (www.wormboss.com.au). Other authors suggest a threshold of 300-500 EPG (based on counts of 10 animals) for treatment of sheep flocks (Coles G.C., personal communication). It is therefore clear that there is a misleading view of FEC thresholds for treatment in sheep and longer term trials justifying these values are lacking. Therefore, to gain maximal information from FEC, strict thresholds for

treatment should not be applied, instead baseline FEC data (i.e. longitudinal data) should be established so that it can be determined when worm burdens deviate for what can be expected on a particular farm. Therefore, besides FEC, accumulated experience of local epidemiological patterns, and knowledge of pastures and grazing history, should be regarded as extremely valuable information to target anthelmintic treatments in small ruminants (Kenyon et al., 2014).

Another area in which FEC can also provide useful information is to indicate levels of pasture contamination, triggering group treatment to reduce the infection pressure, in concert with pasture management regimes. However, this approach is yet to be widely and systematically used in practice, and further research is required (Kenyon et al., 2014).

FEC is of primary importance in determining the efficacy of anthelmintics and monitoring the drug-susceptibility and -resistance status of GIN in small ruminants and other livestock species. There are several methods (e.g. egg hatch assay, larval development assay, molecular methods, etc.) for the detection of anthelmintic resistance (AR) in sheep but the faecal egg count reduction test (FECRT), with its ability to provide a measure of the performance of a number of different anthelmintics at a time, remains the method of choice to monitor anthelmintic efficacy against GIN in livestock. FECRT is currently the only test that can be used to detect resistance to all nematode species and anthelmintics in all hosts (McKenna, 2013). FECRT guidelines are made available by the World Association for the Advancement of Veterinary Parasitology (WAAVP). These guidelines (Coles et al., 1992) provide recommendations on the experimental set up (randomized control trial), sample size (≥ 10 or ≥ 15 animals per treatment group, each excreting at least 150 EPG), the FEC method (McMaster), statistical analysis (FECRT based on the arithmetic mean of grouped FEC after drug administration) and criteria defining reduced efficacy (FECRT $< 90\%$ or FECRT $< 95\%$ and lower limit of 95% confidence interval $< 90\%$).

Different formulae for calculating FECR have been proposed (McKenna, 2002) which differ on the presence of a control group and/or on the use of composite faecal samples.

The following Table 2 (adapted from Roeber et al., 2013) summarizes the main principles and limitation of FECRT.

Table 2. Summary of principles and limitations of FECRT (adapted from Roeber et al., 2013).

| Assay | Principle | Comments and existing limitations | References |
|---------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Faecal egg count reduction test | Provides a an estimate of anthelmintic - efficacy by comparing faecal egg counts from sheep before and after treatment. Resistance is declared if reduction in the number of eggs counted is <95% and the lower confidence interval for the percentage of reduction is below 90%. | <ul style="list-style-type: none"> - Does not accurately estimate the efficacy of an anthelmintic to remove worms. - It rather measures the effects on egg production by mature female worms. - Different anthelmintics require sample collection at different time intervals. - No agreed standard for FEC method or for the calculation of reduction. - Results can be inconclusive due to low analytical sensitivity of the technique. - Different results in repeated experiments. - Does not provide species specific information if undifferentiated. Larval culture required for further differentiation. | <p>Martin et al. (1985)</p> <p>Presidente (1985)</p> <p>Coles et al.(1992)</p> <p>Jackson (1993)</p> <p>Grimshaw et al. (1996)</p> <p>McKenna (1996, 1997, 2006)</p> <p>Taylor et al.(2002)</p> <p>Coles et al.(2006)</p> <p>Miller et al.(2006)</p> <p>Dobson et al.(2009)</p> <p>Levecke et al.(2011)</p> |

Since the publication of the WAAVP guidelines on how to conduct a FECRT, some limitations have been noted, including (i) the ignorance of host-parasite interactions that depend on animal and parasite species, (ii) their feasibility under field conditions, (iii) appropriateness of study design, and (iv) the high detection limit of the recommended FEC method. Field and computer-based studies by Levecke et al. (2011, 2012) highlighted that the interpretation of the FECRT is affected by a complex interplay of various factors, including the mean and level of aggregation of FEC.

6. LIMITATIONS OF COPROMICROSCOPIC TECHNIQUES

The abundance and distribution of GIN in small ruminants is a complex and dynamic issue affected by a whole range of parameters related to the parasite, the host and the environment. The situation is further complicated by interacting regional, seasonal and host-specific factors that influence infection and the fact that helminthoses are usually seen in animals that have concurrent multi-species infections (Morgan et al., 2013).

Although widely used for diagnosis of GIN and other parasites of animal and humans, it is well known that copromicroscopy is prone to a number of shortcomings (Utzinger et al., 2012). First, there is a clear lack of standardization of copromicroscopic techniques and usually each lab uses “its own” method mostly

based on the “lab traditions” rather than on the performance (e.g. sensitivity, specificity, reproducibility, negative predictive value), or operational characteristics (e.g. simplicity, ease of use, user acceptability) of the technique (Rinaldi and Cringoli, 2014). From a general point of view, when choosing a diagnostic technique the following principles should be considered: *“To be useful, diagnostic methods must be accurate, simple and affordable for the population for which they are intended. They must also provide a result in time to institute effective control measures, particularly treatment”* (Banoo et al., 2010). A key point to consider is that different factors may influence the performance of any copromicroscopic technique, especially those based on flotation (e.g. McMaster, Wisconsin, FLOTAC and Mini-FLOTAC). These can include the method of faecal sampling, faecal storage, the duration of faecal storage before analyses, the selection of the flotation solution, and many other laboratory factors.

Second, the results of any copromicroscopic technique strongly depend on the accuracy of laboratory procedures but also on the experience of the laboratory technicians reading the microscopic fields. Hence, a good diagnosis requires in-depth training for faecal sampling, specimen preparation, and experience for subsequent FEC. The “human” factor (i.e. the hands and eyes of technicians) is of fundamental importance for copromicroscopic analyses compared to other diagnostic approaches (i.e. immunological or molecular methods).

Third and most importantly, the main limitation of copromicroscopy is the time and cost to conduct copromicroscopic analysis (in particular FEC) on a representative number of animals. The number of animals to test and frequency of sampling for the FEC performed on faecal samples taken from single animals are seldom informative (Sargison, 2013). In small ruminants, GIN egg counts are generally performed on samples taken from 10/20 animals within a group, and usually show standard deviations that are similar to the arithmetic mean values. Thus, the individual FECs of animals within groups with a mean FEC of 450 EPG might be 50 or 1000 EPG, neither of which provides valid information about the level of challenge to the individual or to the group or about the need for

anthelmintic drug treatment (Sargison, 2013). Also, FEC have been criticized as a treatment indicator because it is poorly correlated with GIN worm burden and animal performance (Sargison, 2013).

A further important point to consider is related to the international economic crisis and the resulting decline of research funds that impose the need to resolve issues at considerably lower costs taking into account the logistical difficulties in conducting field surveys (e.g. cross-sectional and longitudinal surveys of GIN in small ruminants as well as studies on efficacy of anthelmintics). The cost of individual FEC is often too high for small ruminant production, can be attenuated by performing FEC on pooled samples, in which equal amounts of faeces from several individuals are mixed together and a single FEC is used as an index of group mean FEC (Morgan et al., 2005).

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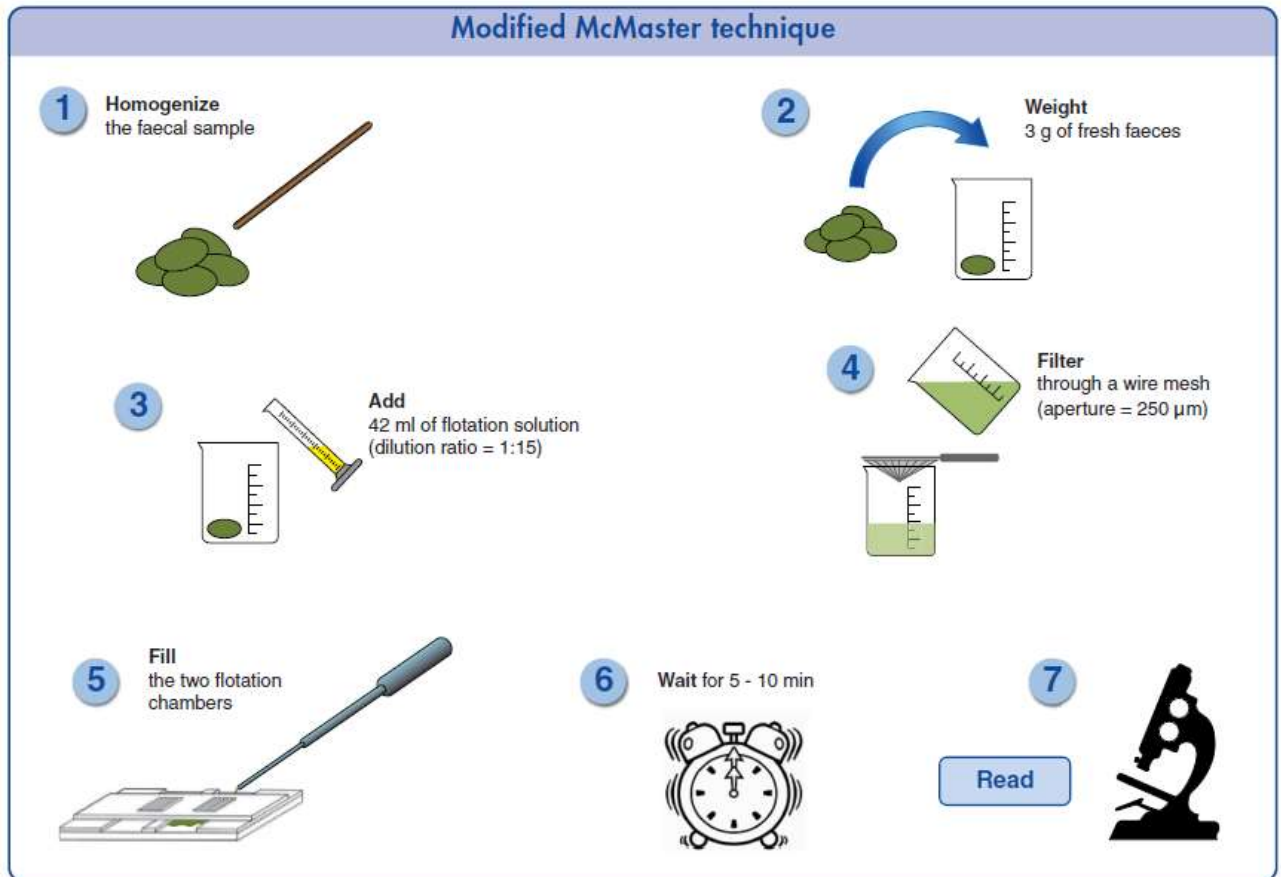
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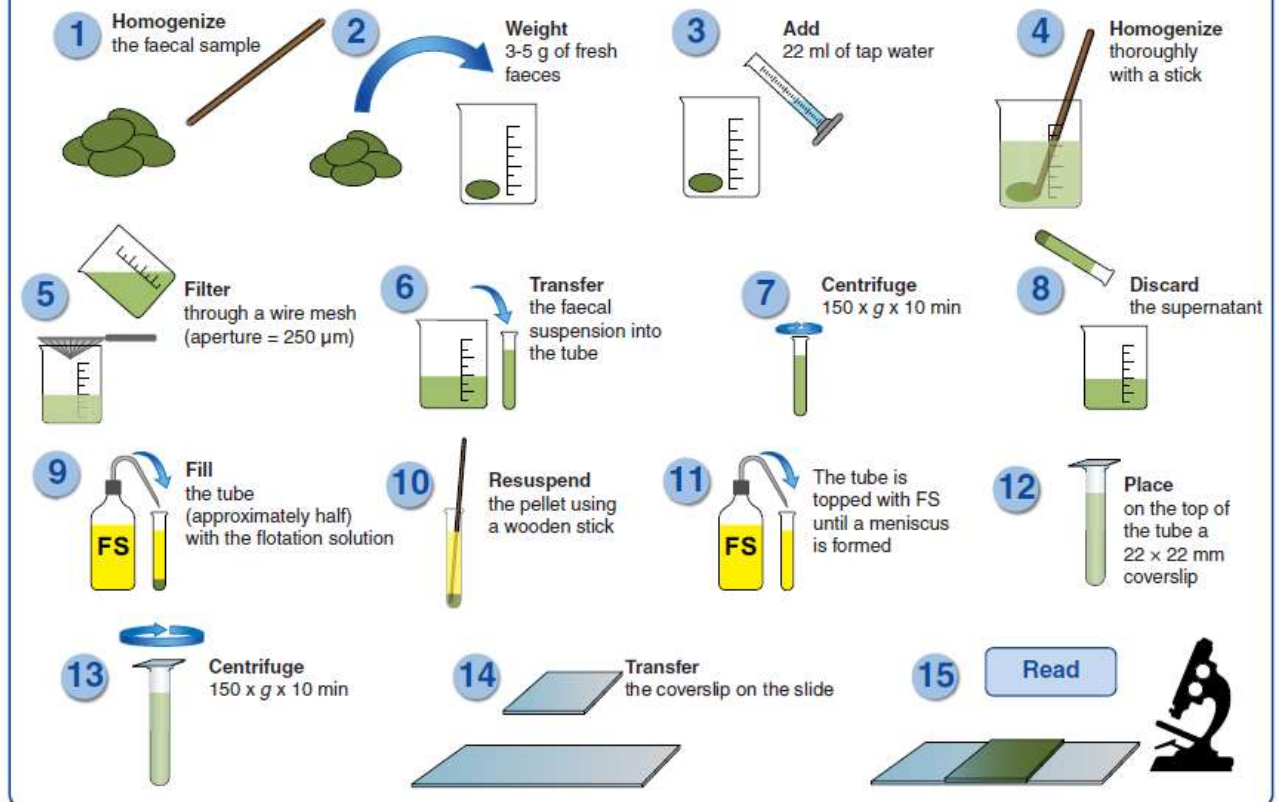
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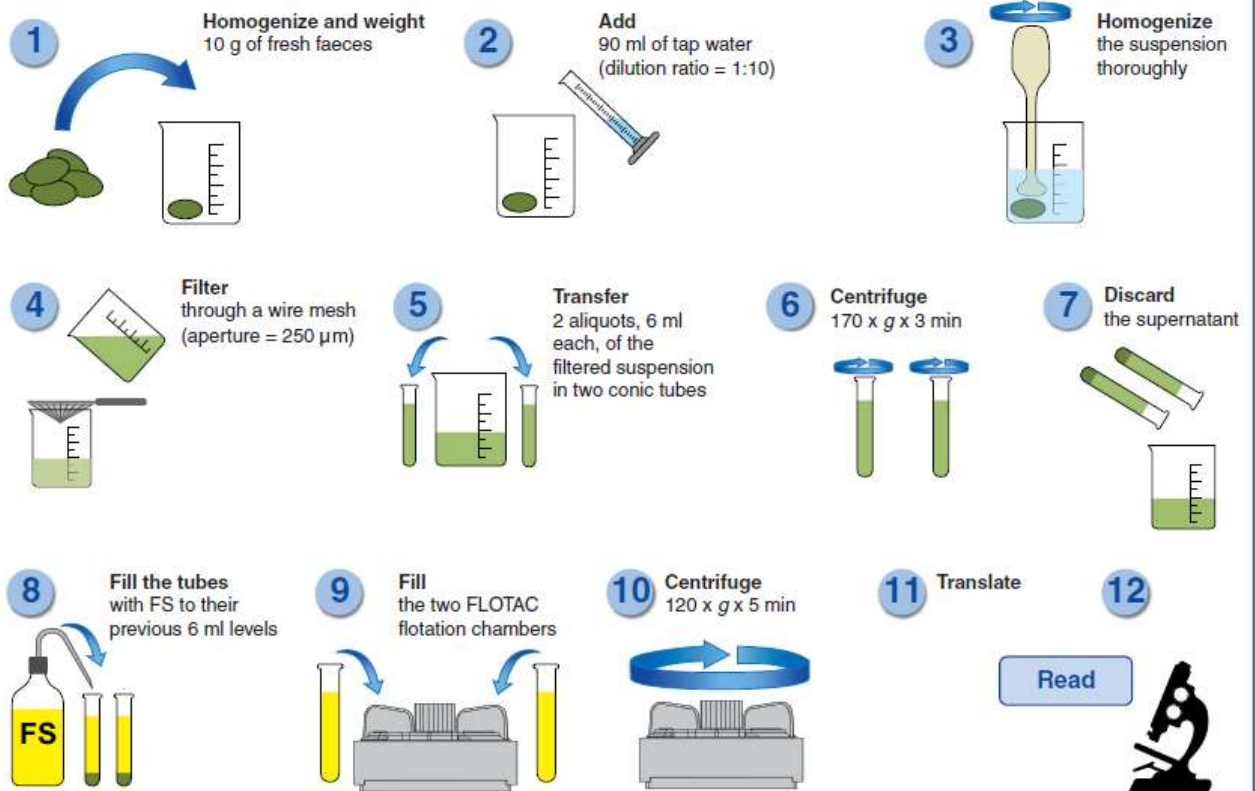
CHAPTER 1 - APPENDIX



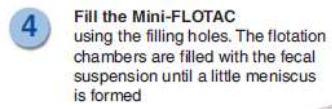
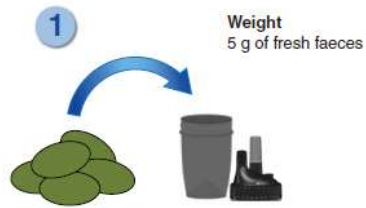
Wisconsin method



FLOTAC dual technique



Mini-FLOTAC technique



OBJECTIVES

The general objective of my PhD was to study into details the different aspects concerning the coprological diagnosis of gastrointestinal nematode (GIN) infections in small ruminants.

The specific objectives were:

1. To define the accuracy of the FLOTAC technique and to compare it with other coprological techniques. For this purpose, laboratory trials were conducted on sheep faecal samples to calibrate the FLOTAC and to compare the diagnostic accuracy of three techniques: simple flotation, McMaster and FLOTAC. The aim was to find the best flotation solution (FS) and to evaluate the influence of faecal preservation methods combined with FS on GIN egg counts.

[Chapter 2].

2. To study the importance of the sampling period and sampling time for the coprological diagnosis of GIN infections in small ruminants. For this purpose, a longitudinal study on GIN faecal egg count (FEC) was conducted in dairy goats aimed at evaluating: the effect of hour of faecal sample collection on GIN FECs and the relationship between FECs and worm burdens.

[Chapter 3].

3. To evaluate the maintenance of anthelmintic efficacy in sheep in a Mediterranean climate. For this purpose, the presence of anthelmintic resistance was investigated on sheep farms using the FLOTAC technique in order to determine whether management practices in this region have allowed the maintenance of anthelmintic efficacy.

[Chapter 4].

4. To determine the value of pooled faecal samples to assess GIN infection intensity (FEC) and anthelmintic efficacy (FECR). For this purpose, field trials were conducted to: compare FEC and FECR from individual sheep

samples and pools of different size (5, 10 and 20 individual sheep samples); assess the effect of three different analytic sensitivities (10, 15 and 50) on individual and pooled samples using McMaster (analytic sensitivities = 15 and 50) and Mini-FLOTAC (analytic sensitivity = 10) and; determine the effect of the pooling on FECR.

[Chapter 5].

CHAPTER 2

Calibration and diagnostic accuracy of simple flotation, McMaster and FLOTAC for parasite egg counts in sheep*

* Based on the manuscript: Rinaldi, L., Coles, G.C., Maurelli, M.P., Bosco, A., Musella, V., Cringoli G., 2011. Calibration and diagnostic accuracy of simple flotation, McMaster and FLOTAC for parasite egg counts in sheep. Vet Parasitol. 177 (3-4), 345-52.

1. INTRODUCTION

One of the most important issues facing sheep health is the development of anthelmintic resistance, a situation only being rescued in several countries by the introduction of a new anthelmintic type monepantel (Kaminsky et al., 2008). Early detection of resistance to all types of anthelmintic and especially the macrocyclic lactones would be of value so that the necessary changes in management can be made. A sensitive faecal egg count (FEC) procedure combined with use of a discriminating dose should help solve this problem. The parasites are usually diagnosed by copromicroscopic techniques (Cringoli et al., 2004; Mes et al., 2007). FEC techniques are considered relatively straightforward and protocols such as the McMaster technique and the Wisconsin flotation technique have been available for many years (Cringoli et al., 2010).

The different variants of the McMaster method (MAFF, 1986) have the advantage that they are quick to use, particularly if centrifugation is not included in the protocol.

For most purposes its sensitivity of 50 or 25 eggs per gram of faeces (EPG) is adequate. However, it is not suitable for helminths such as flukes and for situations where sensitive egg counts or lungworm larval counts are required (Cringoli et al., 2010; Duthaler et al., 2010; Rinaldi et al., 2010). FLOTAC is a multivalent sensitive and accurate copromicroscopic method of examining faecal samples for the presence of eggs, larvae, oocysts and cysts. This technique uses the FLOTAC apparatus which allows up to 1 g of faeces to be prepared for microscopic analysis (Cringoli, 2006; Cringoli et al., 2010) (Fig 1). Flotation solutions (FS) and faecal preservation methods have fundamental role in determining the analytic sensitivity, the precision, and the accuracy of any copromicroscopic technique, either qualitative or quantitative, based on flotation, including the FLOTAC technique (Cringoli et al., 2004, 2010). In view of these considerations, there is a need for detailed calibration of the FLOTAC and other FEC techniques, to determine the optimal FS and faecal preservation method for an accurate diagnosis of parasitic elements. The present study was aimed at carrying out a calibration and a comparison of diagnostic accuracy of three FEC techniques, the simple flotation technique (MAFF, 1986), the McMaster (MAFF, 1986) and FLOTAC (Cringoli et al., 2010), in order to find the best FS for *Dicrocoelium dendriticum*,

Moniezia expansa and gastrointestinal (GI) strongyles, and to evaluate the influence of faecal preservation methods combined with FS on egg counts.

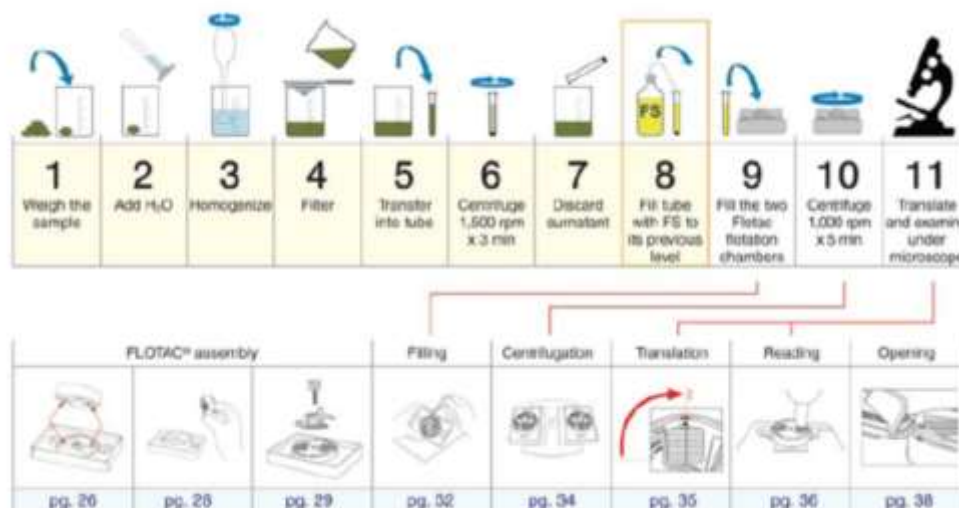


Fig. 1. The Basic steps of the FLOTAC Technique (analytic sensitivity: 1 EPG, 1 LPG, 1 OPG and 1 CPG).

2. MATERIAL AND METHODS

2.1. Experiment 1—calibration of flotation solutions and faecal preservation methods

To determine the optimum FS, faecal preservation method, and technique for counting helminth eggs, faecal samples from naturally infected sheep were collected, combined, thoroughly homogenized and divided into four aliquots of 120 g each. These were either directly examined (i.e. fresh), or preserved in 5 or 10% formalin or frozen at -30°C prior to counting. Formaldehyde was added at 3 parts fixative to 1 part faeces. To prepare samples for examination by three counting techniques: (i) simple flotation technique (MAFF, 1986), (ii) McMaster technique (MAFF, 1986) and (iii) FLOTAC technique (Cringoli et al., 2010), each aliquot was diluted with 9 parts of water or water plus formalin (i.e. faecal dilution of 1:10), thoroughly homogenised and filtered through a 250 μm wire mesh sieve. The filtered suspension was divided into 162 aliquots of 6 ml to have six replicates for each of 9 FS for the 3 techniques. After centrifugation at 1500 rpm (170 g) for 3

min supernatant was discarded and flotation solutions were added. Tubes were randomly assigned to the three techniques and to the 9 FS described in Table 1. For the simple flotation technique tubes were filled with FS to give a slight meniscus and a 18 mm × 18 mm cover slip was added and left for 15 min before being removed and all eggs counted.

Table 1. Flotation solutions used for the calibration and comparison of the three techniques: McMaster, simple flotation technique and FLOTAC.

| Flotation solutions | | Specific gravity (s.g.) |
|---------------------|-------------------------------------------|-------------------------|
| FS1 | Sheather's Sugar Solution | 1.200 |
| FS2 | Saturated Sodium Chloride | 1.200 |
| FS3 | Zinc Sulphate | 1.200 |
| FS4 | Sodium Nitrate | 1.200 |
| FS5 | Sucrose and Potassium Iodomercurate | 1.250 |
| FS6 | Magnesium Sulphate | 1.280 |
| FS7 | Zinc Sulphate | 1.350 |
| FS8 | Potassium Iodomercurate | 1.440 |
| FS9 | Zinc Sulphate and Potassium Iodomercurate | 1.450 |

For examination by the McMaster technique (special modification of the McMaster method—MAFF, 1986), FS were added up to 6 ml, the contents of the tube thoroughly mixed and 1.0 ml was then taken up by pipette to load the two cells of the McMaster slide (Weber Scientific International, England; volume = 1.0 ml). Slides were allowed to stand for 10 min before reading both cells. One egg seen is equivalent to 10 eggs per gram of faeces (analytic sensitivity = 10 EPG).

For the FLOTAC technique, FS were added up to 6 ml, the contents thoroughly mixed and used to fill one of the two chambers of the FLOTAC-100 (volume of each chamber = 5 ml). Thus, a single flotation chamber of the FLOTAC-100 was utilized for each replicate (analytic sensitivity = 2 EPG). After centrifugation of the FLOTAC apparatus at 1000 rpm (120 g) for 5 min, the top of the flotation chambers were translated and the number of eggs counted.

2.2. Experiment 2—preservation by vacuum packing

Experiment 2 was aimed at determining the applicability of vacuum packing as faecal preservation method for GI strongyle FEC by FLOTAC and McMaster (using

FS2) (Fig. 2). It should be noted that we focussed this experiment on GI strongyles in order to contribute to the ongoing debate on the FEC reduction test. Faecal samples from naturally infected sheep were collected, combined, thoroughly homogenized and divided into 13 aliquots of 30 g each. These were either directly examined at day zero (i.e. fresh, D0), or preserved by vacuum packing at room temperature and examined weekly for 28 days (VP-RT: D7, D14, D21, D28), or preserved by vacuum packing in the fridge (+4 °C) and examined weekly for 28 days (VP-F: D7, D14, D21, D28), or preserved in the fridge (+4 °C) without vacuum packing and examined weekly for 28 days (F: D7, D14, D21, D28). Vacuum packing was performed using a domestic appliance; this method can be used for preserving samples (van Wyk, J. personal communication).



Fig. 2. The vacuum packing to preservation the faecal samples

2.3. Statistical analysis

The arithmetic mean eggs per gram of faeces (EPG), standard deviation (SD), and Coefficient of Variation (CV) of EPG values were calculated for the different FS for

each preservation method and each technique. Differences between solutions were analyzed using an one-way ANOVA with post hoc Fisher's least significant difference (LSD). Statistical analysis was carried out using STATA 10.0 software (Stata Corp., TX 77845, USA). In addition, a likelihood ratio test of the equality of the CV of k normally distributed populations was performed using software developed by the Statistical Services at the Forest Products Laboratory (USA; <http://www1.fpl.fs.fed.us/covtestk.html>).

3. RESULTS

3.1. Experiment 1—calibration of flotation solutions and faecal preservation methods

The results of the experiment 1 are shown in Figs. 1–3 which respectively shows *D. dendriticum*, *M. expansa* and GI strongyle egg counts (EPG and CV) in the composite sheep faecal sample, stratified by diagnostic technique, FS and faecal preservation method. The “gold standard” FS was defined as the FS which produced the highest EPG and the lowest CV. Statistical comparisons were performed only for FS producing EPG above the 50% of the gold standard (marked with a blue line in Figs. 3–5).

3.1.1. *Dicrocoelium dendriticum*

Eggs of *D. dendriticum* floated only with flotation solutions from FS6 to FS9 for all the three techniques used. The best results for *D. dendriticum* were obtained with FLOTAC using FS7 (EPG = 219, CV = 3.9%), FS8 (EPG = 227, CV = 5.2%) and FS9 (EPG = 210, CV = 5.3%) (no significant difference between FS) on fresh faeces (Fig. 1). F7 (EPG = 108, CV = 31.7%) was less effective when using McMaster but F8 (EPG = 188, CV = 16.3%) gave acceptable results although with high CV. The simple flotation performed very poorly for estimating numbers of *D. dendriticum* eggs.

With respect to faecal preservation methods, FLOTAC with FS8 also performed well with faecal samples preserved in 5% and 10% formalin or if frozen, but less so for FS7 and FS9.

3.1.2. *Moniezia expansa*

All nine FS were capable of bringing *M. expansa* eggs in flotation for all the three techniques used.

The “gold standard” for *M. expansa* was obtained with FLOTAC, using FS3 (EPG = 122, CV = 4.1%) on fresh faeces but there were no relevant differences between the different FS. FLOTAC also showed good results with FS3 with faecal samples preserved in 5% formalin, whereas with the other two preservation method, i.e. 10% formalin and frozen, controversial results were obtained regarding FS. As regard to McMaster, the best results were obtained with FS6 on fresh faeces (EPG = 120, CV = 39.1%). Also for this method there were no significant differences between the different FS, however, CV was higher than FLOTAC for all FS and preservation methods. As regard to simple flotation, the mean EPG were lower and the CV were higher than with the other two techniques for all the FS and faecal preservation method used.

3.1.3. *Gastrointestinal strongyles*

Both the FLOTAC and McMaster techniques gave acceptable counts on fresh faeces with FS1 to FS6 but usually with a lower CV with FLOTAC counts. The “gold standard” for GI strongyles was obtained with FLOTAC when using FS5 (EPG = 320, CV = 4%) and FS2 (EPG = 298, CV = 5%).

With all three methods of preservation using formalin or freezing satisfactory egg counts were not obtained by any counting method. Moreover, as with egg counts of *M. expansa* and *D. dendriticum* the results from simple flotation were unacceptably low (Fig. 5).

3.2. Experiment 2—preservation by vacuum packing

Anaerobic storage by using vacuum packing of faecal samples and refrigeration at 4 °C permitted egg counts up to 21 days after collection although after this time some egg structure began to disappear (Fig. 6). Mould formed by day 14 if refrigeration without vacuum packing was used limiting the acceptability of this method. Preservation by vacuum packing with storage at room temperature (21.8 °C) was satisfactory until day 21 but from day 7 the smell from the samples limited their acceptability. From day 14 there was an increase in larvation of the eggs.

4. DISCUSSION

If a simple fast decision is required on whether sheep should be treated for infection with GI strongyles, *M. expansa* or *D. dendriticum* the present data confirms that the McMaster technique or its on farm version, FECPAK (Presland et al., 2005), are satisfactory, although the best results, in terms of sensitivity, accuracy and repeatability was obtained with FLOTAC technique, as in previous studies made for different parasites of veterinary and human importance (Cringoli et al., 2010), including parasites of sheep (Rinaldi et al., 2010). The simple flotation technique should never be used due to the very low and variable results obtained. The lower sensitivity, accuracy and repeatability of McMaster technique for egg count may have been also mentioned by Mes et al. (2007), who have reported that this technique requires extrapolation, and thus it renders EPG estimates less precise than methods that do not require extrapolation, such as the Wisconsin flotation method (Cox and Todd, 1962; Egwand and Slocombe, 1982) and the modified salt-sugar flotation method (Mes et al., 2007). Following this line of thought, larger multiplication factors are needed for extrapolation, for example, under the smaller McMaster slide areas (volumes), the less precise EPG counts will result. Moreover, using the FLOTAC technique, a large amount of faecal suspension is examined, and so also the sensitivity is greater; thus, this technique is less likely to give false negative results. The results confirm, also, that the faecal preservation methods as flotation solutions (FS) have a fundamental role in determining the analytic sensitivity of any copromicroscopic techniques; it is noteworthy if a

sample has examined to be fresh, does not produce the same results if the method of faeces preservation changes (e.g., frozen, fixed in formalin or in other fixatives). Faecal samples containing *M. expansa* or *D. dendriticum* eggs can be stored chemically (formaldehyde) or by freezing but this should not be used with GI strongyle eggs as demonstrated also by Foreyt (1986) for nematoda eggs in deer.

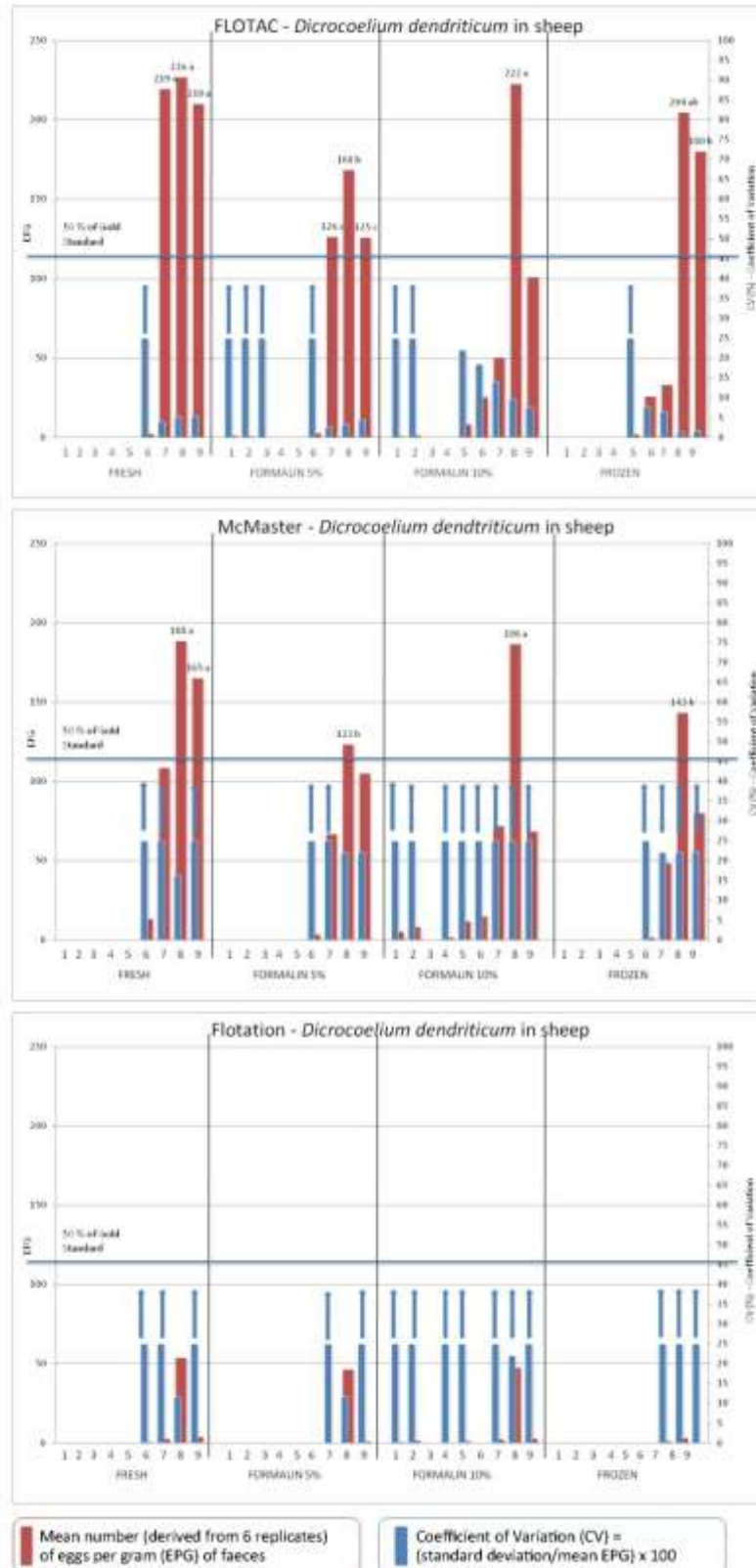


Fig. 3. The recovery of eggs of *D. dendriticum* from sheep faeces by FLOTAC, McMaster and tube flotation using 9 different flotation solutions and 4 different methods of sample preservation. *P < 0.05; significant differences for different letters.

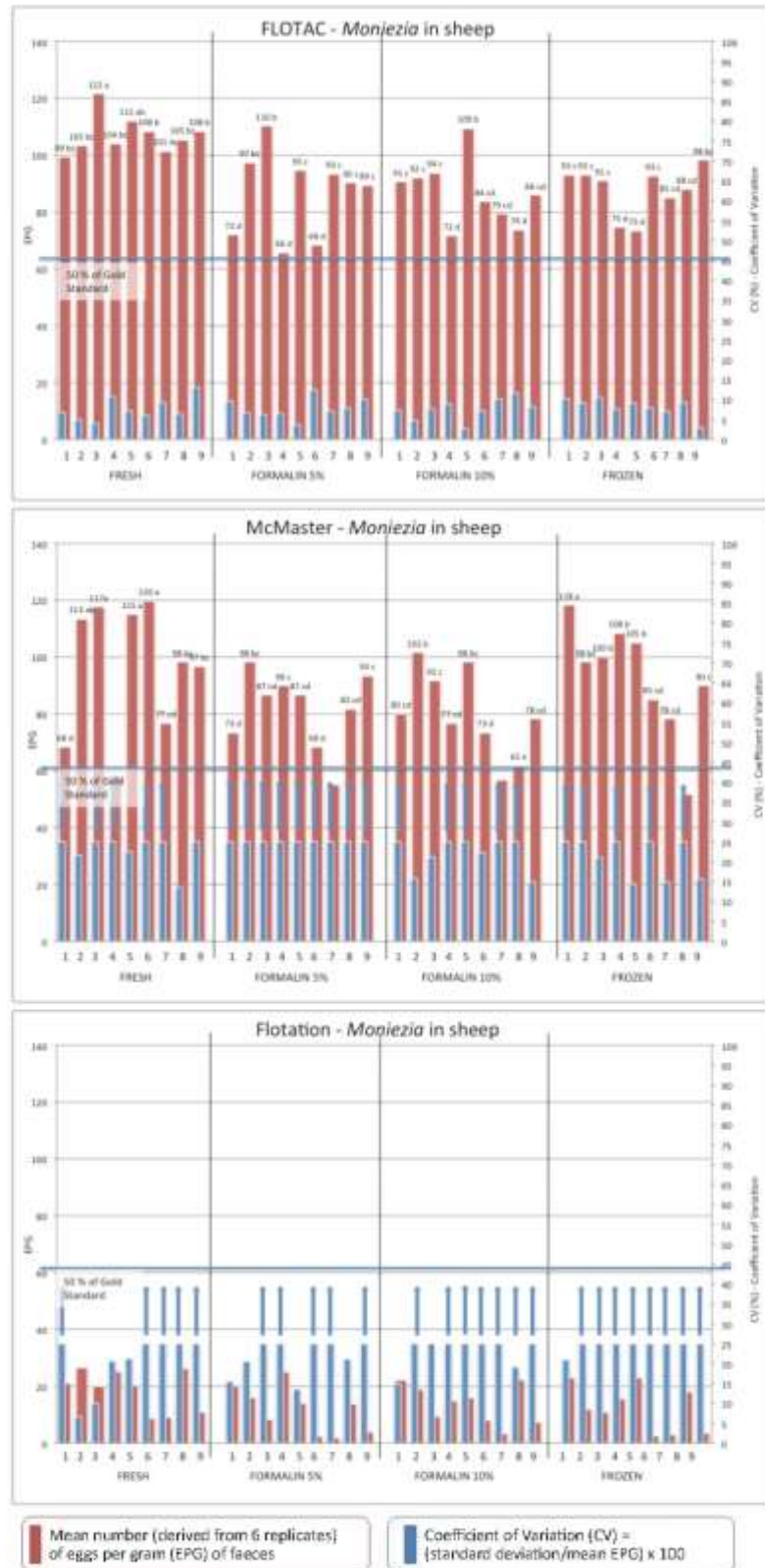


Fig. 4. The recovery of eggs of *M. expansa* from sheep faeces by FLOTAC, McMaster and tube flotation using 9 different flotation solutions and 4 different methods of sample preservation. * $P < 0.05$; significant differences for different letters.

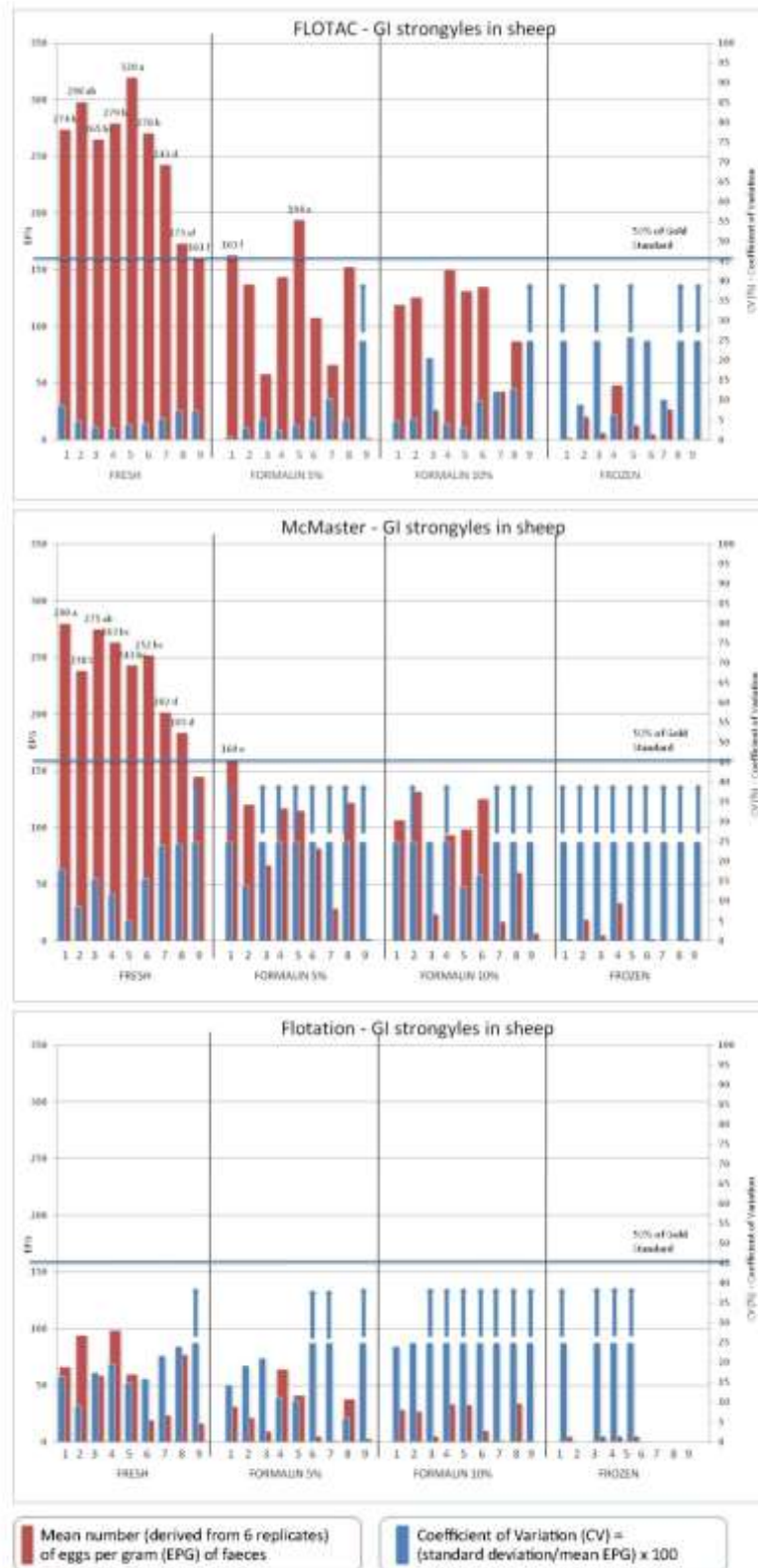


Fig. 5. The recovery of eggs of GI strongyles from sheep faeces by FLOTAC, McMaster and tube flotation using 9 different flotation solutions and 4 different methods of sample preservation. *P < 0.05; significant differences for different letters.

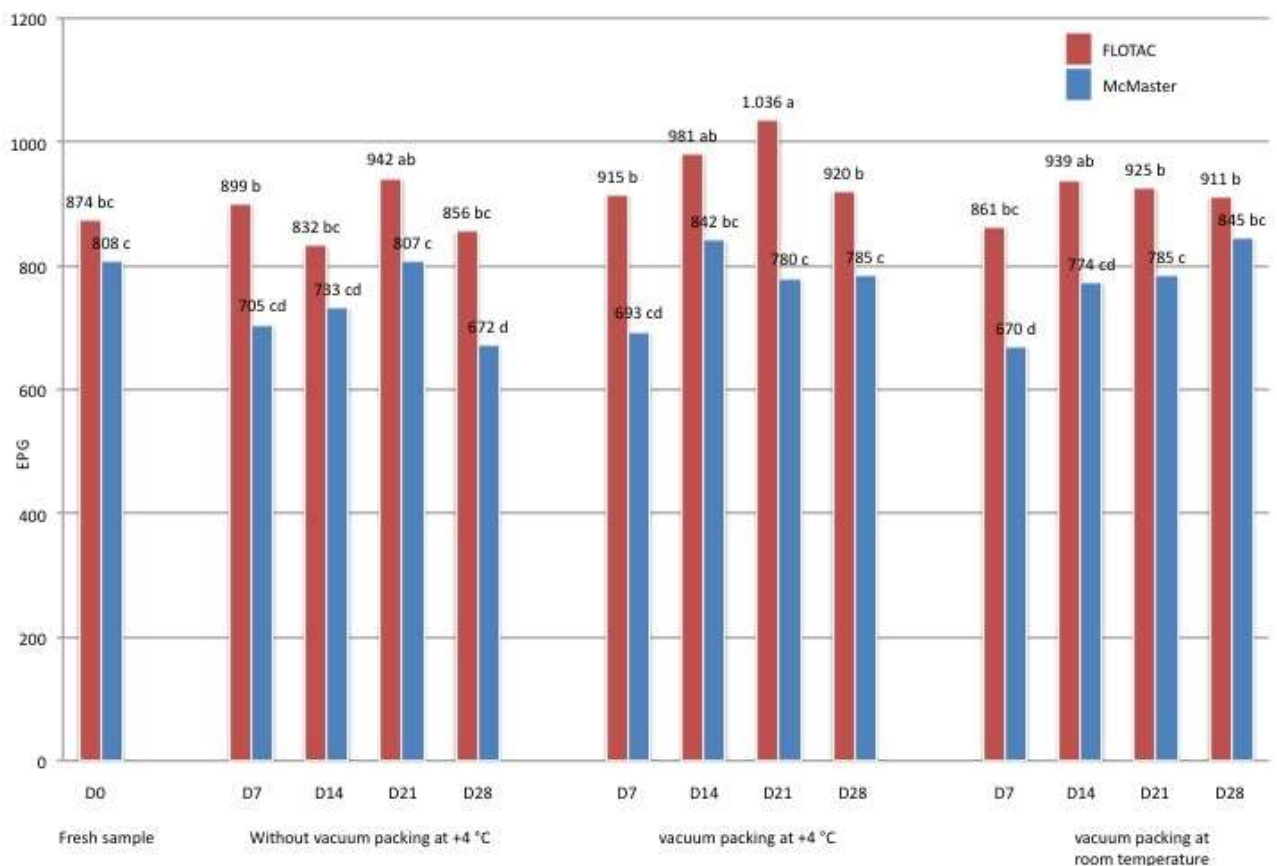


Fig. 6. GI strongyle egg counts using McMaster or FLOTAC counts in fresh faecal samples or stored by refrigeration, vacuum packing or refrigeration and vacuum packing for up to 28 days (D7, D14, D21, D28).

Vacuum storage or refrigeration can be used to store faecal samples for up to 21 days without significantly reducing the egg counts, although the best combination is both vacuum packing and refrigeration, and so it could be a good alternative method to preserve the faeces to analyze and should be tried also on other helminth eggs of other animal species. Whilst this could be of value if very large numbers of samples are collected at one time or samples are being transported long distance for analysis, the storage is obviously not relevant if rapid decisions on treatment are required. Faecal egg counts have two important purposes. The first is the decision of whether to treat to improve the health of the animals and/or reduce pasture contamination. This decision is complicated by different egg laying rates with different nematodes, e.g., *Haemonchus contortus* versus *Teladorsagia*

circumcincta, egg counts unrelated to worm burdens, e.g., *Nematodirus* sp. and the physiological/immunological status of the host, e.g., resilient versus 'normal' versus resistant animals. So as yet there is no agreed egg count data for deciding when to treat and it may vary from animal to animal in the flock thus giving rise to the concept of targeted selective treatment (Kenyon et al., 2009) in which refugia is promoted by leaving selected animals untreated. Methods that are not too accurate are fully acceptable for these egg counts. The second use of egg counts is for the detection of anthelmintic resistance. To improve the sensitivity of detection accurate egg counts are required. Whilst counting of ten samples at treatment and ten samples 7–14 days later per anthelmintic used may be valuable for a research project the expense involved will not be acceptable to sheep farmers with very low profit margins. To overcome this composite sampling must be used and accurate egg counts have to be performed to give a reliable indication of the degree of resistance. This may be to check the efficacy of quarantine dosing to avoid the introduction of resistant worms when animals are brought on to a farm. It may also be to establish the resistance status of a flock so that evasive strategies may be introduced before resistance becomes a serious practical problem with any particular anthelmintic and results in lost production. Coles et al. (1992) suggested that efficacies in the faecal egg count reduction test of less than 95% with 95% confidence level less than 90% will indicate the presence of resistance. This, of course, requires the counting of samples from several sheep. Our ongoing research suggests that these values are incorrect and too low and that by using very accurate egg counts early stages of anthelmintic resistance can be detected. If confirmed this could remove the need to have molecular based tests to detect the early emergence of resistance, so far a largely elusive goal particularly for macrocyclic lactones. The key issue for counting helminth eggs for the detection of resistance is the use of a highly sensitive, accurate and repeatable egg counting procedure. This applies to other grazing species as well as sheep, including cattle (unpublished) and ivermectin resistance in horses (Dudeney et al., 2008). The present data confirms that the FLOTAC meets these requirements for use in accurate repeatable egg counts with sheep and could become the counting method of choice for all investigations into the epidemiology of anthelmintic resistance in all animal species including humans.

Although best results are often obtained with flotation solutions containing mercury salts the toxicity of these chemicals and the strict legal requirements for their disposal will exclude their widespread use (Cringoli et al., 2010). Based on the present results and previous results by Rinaldi et al. (2010) we suggest the use of two solutions that are easy and cheap to purchase and prepare, saturated sodium chloride (FS2) for nematoda and cestoda eggs and saturated zinc sulphate (FS7) or zinc chloride for trematoda eggs and nematoda larvae. The importance of *M. expansa* in lamb growth is debatable (Elliott, 1986) but since FS7 floats *Fasciola hepatica* and other trematode eggs, FLOTAC should be very useful in the detection of anthelmintic resistance in fluke which appears to be a growing problem with triclabendazole (Fairweather, 2009) as well possibly occurring with closantel (Fairweather and Boray, 1999). Further research is likely to result in the use of FLOTAC in the counting of oocysts, e.g., *Eimeria* sp. and also the detection of anti-coccidial resistance.

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CHAPTER 3

Is gastrointestinal strongyle faecal egg count influenced by hour of sample collection and worm burden in goats?*

*Based on the manuscript: Rinaldi, L., Veneziano, V., Morgoglione, M.E., Pennacchio, S., Santaniello, M., Schioppi, M., Musella, V., Fedele, V., Cringoli, G., 2009. Is gastrointestinal strongyle faecal egg count influenced by hour of sample collection and worm burden in goats? *Vet. Parasitol.* 163, 81-86.

1. INTRODUCTION

The issue of controlling gastrointestinal (GI) strongyles is of particular economic importance in goat production system worldwide (Rinaldi et al., 2007b,c). Conventional methods of worm control involve treatment(s) of the whole flock with synthetic anthelmintics. However, in this day and age, the global problem of anthelmintic resistance in small ruminants ensures that attention also needs to be given to the sustainability of anthelmintic treatment regimes as well as to their immediate economic benefit (Cringoli et al., 2007a,b, 2008). There is currently a general agreement to replace the practice of treating the whole flock with targeted selective treatments (TST), where only animals showing clinical symptoms or reduced productivity are given drugs (van Wyk et al., 2006). Different pathophysiological and performance indicators of GI strongyle infection in small ruminants have been recently proposed; for example, the FAMACHA system (van Wyk and Bath, 2002) can be used to identify goats suffering from anaemia (likely caused by *Haemonchus*), and a diarrhoea index can be a good indicator of actual nematode infection during the summer and autumn in a temperate climate (Cabaret, 2004). In addition, body condition scoring (van Wyk and Bath, 2002), and liveweight gain and milk production (Hoste et al., 2002a,c) are also potential methods for identifying animals requiring anthelmintic treatments. However, the value of these methods in different climates and the benefits to animal productivity need further research (Ketzis et al., 2006) and faecal egg count (FEC) techniques remain the most common approaches for the estimation of prevalence and intensity of GI strongyle infections (Cringoli et al., 2004; Villanua et al., 2006). FEC results may be affected by many factors, either depending on FEC techniques or depending on biological factors. There is a clear need for the standardization of FEC techniques (Cringoli et al., 2004), and recently, the Flotac techniques (Cringoli, 2006) has been developed for sensitive and accurate FECs. The validity of these techniques are supported by studies for different parasites in different host species (Rinaldi et al., 2007a,b,c; Gaglio et al., 2008; Traversa et al., 2008; Utzinger et al., 2008; Knopp et al., 2009) including GI strongyles of ruminants (Cringoli G, unpublished data; FLOTAC1 Manual – Flotac techniques - Herbivores). Concerning the biological factors, FEC may be subjected to a great within-individual variation due to factors such as host reproductive status, weather, season, random day-

today variation, and the phase of the parasitic infection (Villanua et al., 2006). In order to study the factors that can influence the significance of FEC results, the present paper reports a longitudinal study on GI strongyle FEC in dairy goats aimed at evaluating: (i) the effect of hour of faecal sample collection on GI strongyle FECs and (ii) the relationship between FECs and worm burdens.

2. MATERIALS AND METHODS

2.1. *Study farm and study animals*

The study was conducted at the experimental farm of the “C.R.A., Unita` di Ricerca Zootecnia Estensiva, Bella Scalo, Muro Lucano”, located in the Potenza province of southern Italy (40°21'N and 15°30'25"E) at 360 m above sea level. A total of 63 female Siriana goats were used for the study. They were approximately 1.5 years old at the start in July, 2005, and in their second grazing season. The goats in the farm grazed for 8 h/day and were supplemented with concentrates, corresponding to 50% of energy requirements. These goats, together with the rest of the flock, had been treated with moxidectin in June. Before the start of the study each goat was randomly assigned to a sampling day (see below).

2.2. *Relationship between the hour of sampling and GI strongyle FEC*

Every 3 weeks from 13th July 2005 to 6th September 2006 fecal samples were collected every 2 h for 24 h from three of the goats. At each sampling day, three goats were individually housed in digestibility cages containing a sieve in the bottom for separating faeces, as described by Fedele et al. (2002). The three cages were placed in the box where the rest of the flock was housed during the night after grazing. It should be noted that in order to avoid possible bias due to the caging, the experimental goats were acclimatized to the cages each evening for 1 week before each sampling day. Goats were fed with hay and concentrates when caged. In addition, a soft lamp illuminated the stable so that goats were not disturbed by the technicians during the faecal sample collection. Thus, in the present study we attempted to avoid any confounding factor, being aware, however, that the change of diet resulting from grazing pasture to being confined in the box may have influenced the results. Every 3 weeks for 14 months faeces were collected every 2 h for 24 h from the individually caged goats. The faeces

were those passed by the goats during the 2 h preceding each collection. For each goat a 10 g sample obtained from this material, thoroughly homogenized, was analyzed using the Flotac double technique (Cringoli, 2006; FLOTAC1 Manual - Herbivores) having an analytic sensitivity of 2 EPG; a sucrose-based solution (specific gravity = 1.250) was used as flotation medium. To explore relationships between FECs and adult parasite counts, on the day following sampling, the three goats were euthanized and the nematodes present in the abomasa and intestines were recovered, identified and counted. Mean, standard error and 25th, 50th and 75th percentiles of GI strongyle EPG values (not ln-transformed) were calculated for each sampling hour interval, pooling the data from the 63 goats. Then, EPG values were ln-transformed in order to achieve the Normal distribution, as detected by the analyses of the Normality tests of Shapiro–Wilk ($P > 0.05$) and the Normal Q–Q Plots. On these ln-transformed data a generalized linear model (GLM) for the analysis of the effect of hour of sample collection on GI strongyle FEC was performed. In particular, EPG values were introduced in the model as dependent variables, hour and month of sampling as categorical fixed factors and individual animal was entered in the model as a random factor. All the statistical analyses were performed using SPSS software (Version 13).

2.3. Relationship between worm burden and FEC

On each sampling day, in order to have a FEC representative of the 24 h for each goat, a 5 g composite sample from the 12 samples was analyzed using the Flotac double technique as described above. In addition, on each sampling day a composite faecal culture was made per each goat (MAFF, 1986). Third stage larvae were identified using the morphological keys proposed by Gevrey (1971) and van Wyk et al. (2004). When a coproculture had 100 or less third stage larvae, all were identified; when more than 100 larvae were present, only 100 were identified. On the day following faecal sampling, the three goats were euthanized, and adult nematodes in the abomasa and intestines were recovered, identified and counted. The viscera were processed for sample collection, further worm counts and identification of parasites present in the abomasum and small and large intestines, following the procedures described in the WAAVP guidelines for evaluating the efficacy of anthelmintics in ruminants (Wood et al., 1995). Mean and standard

error of females, males and EPG counts were calculated for each GI strongyle species based on pooling data from the 63 goats. The degree of aggregation of FECs and worm counts of GI strongyles was assessed using the parameter k from the negative binomial distribution. Worm count values and EPG were ln-transformed in order to achieve the Normal distribution, as detected by the analyses of the Normality tests of Shapiro–Wilk ($P > 0.05$) and the Normal Q–Q Plots. On these ln-transformed data, for each GI strongyle species, the relationship between worm burden and EPG was evaluated using Pearson correlation. All the statistical analyses were performed using SPSS software (Version 13) and STATA 9.2 software.

3. RESULTS

3.1. Relationship between the hour of sampling and GI strongyle FEC

Table 1 shows the GI strongyle EPG values (arithmetic means) every 2 h over the 24-h sampling period, pooled for the 63 goats. The k values indicate that the data for all EPGs were aggregated (see below). The mean values of GI strongyle EPG ranged from 4417.0 (hour interval 2.00–4.00) to 8652.9 (hour interval 6.00–8.00). The results of GLM (controlling for the effect of individual by considering it as a random effect) did not revealed any significant effect of the hour of sample collection ($F_{11,63} = 0.99$; $P = 0.449$) on FEC, whereas a significant effect of the sampling month (seasonality) was found ($F_{20,63} = 27.5$; $P = 0.000$). Fig. 1 shows the GI strongyle FEC during all the study period; the highest EPG values were observed between April and June.

Table 1. Two-hourly GI strongyle FECs (arithmetic means) over 24 h pooled for 63 goats.

| Time of sampling | Arithmetic mean | Standard error | Percentiles | | |
|------------------|-----------------|----------------|-------------|------|--------|
| | | | 25th | 50th | 75th |
| 8.00-10.00 | 7254.2 | 1800.7 | 1943 | 3331 | 8346 |
| 10.00-12.00 | 6184.2 | 1006.4 | 1883 | 3998 | 7547 |
| 12.00-14.00 | 4838.3 | 965.9 | 143 | 3383 | 4657 |
| 14.00-16.00 | 4947.4 | 866.3 | 1658 | 2982 | 5672 |
| 16.00-18.00 | 6923.2 | 1537.3 | 1974 | 3088 | 7032 |
| 18.00-20.00 | 6816.9 | 1316.8 | 1890 | 3516 | 8856 |
| 20.00-22.00 | 5686.3 | 989.8 | 1904 | 3530 | 8380 |
| 22.00-24.00 | 4449.3 | 793.9 | 1012 | 2640 | 6396 |
| 24.00-2.00 | 6965.3 | 1426.2 | 1025 | 3584 | 7495 |
| 2.00-4.00 | 4417.0 | 855.4 | 1163 | 2932 | 5501 |
| 4.00-6.00 | 8025.1 | 1572.4 | 1634 | 4138 | 9465 |
| 6.00-8.00 | 8652.9 | 1605.8 | 2185 | 4189 | 10,633 |

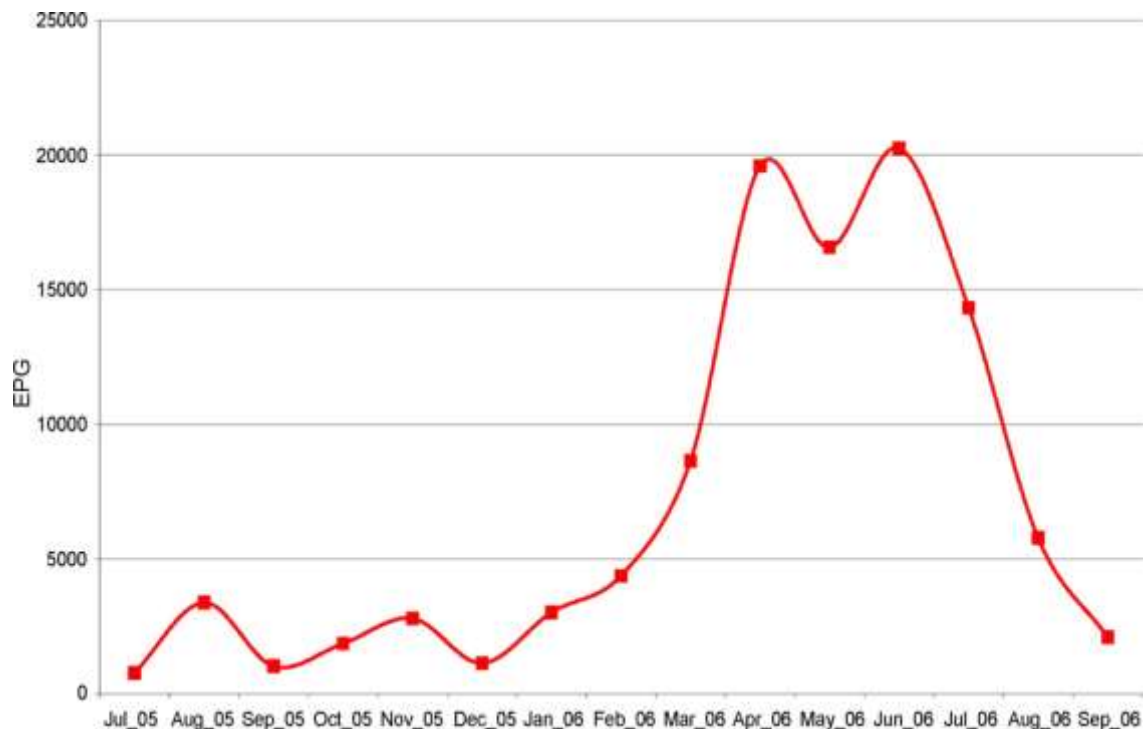


Fig. 1. Monthly GI strongyle FECs (arithmetic means) in the studied goats.

3.2. Relationship between worm burden and FEC

The adult nematodes recovered and identified in the goats were: *Teladorsagia circumcincta* and *Haemonchus contortus* in the abomasum; *Trichostrongylus colubriformis* in the small intestine; and *Oesophagostomum venulosum* in the large intestine. GI strongyle EPG and worm population were aggregated with a positive skewed distribution; this aggregation was also found among the four species of GI strongyles, especially *H. contortus* (see the values of the k parameters from the negative binomial distribution in Table 2). Table 2 also shows the arithmetic mean and standard error of the different species of GI strongyles (EPG, number of females, males and total worms), the female/male ratio and the correlation between EPG and total number of worms (data pooled for 63 goats). The mean number of adult GI strongyles counted in the 63 studied goats was 4447.2 (range = 310–13,992).

Table 2. GI strongyle FECs and adult parasite counts (arithmetic means) pooled for 63 goats.

| Parameters | | GI strongyles | <i>Haemonchus contortus</i> | <i>Oesophagostomum venulosum</i> | <i>Trichostrongylus colubriformis</i> | <i>Teladorsagia circumcincta</i> |
|--------------------------------|-----------------|---------------|-----------------------------|----------------------------------|---------------------------------------|----------------------------------|
| EPG | Arithmetic mean | 7660.8 | 3866.7 | 345.4 | 2490.5 | 958.2 |
| | Standard error | 1287.7 | 753.3 | 79.2 | 406.9 | 146.2 |
| | k parameter* | 0.65 | 0.70 | 0.70 | 0.70 | 0.68 |
| Females | Mean | 2844.4 | 555.4 | 17.5 | 1725.9 | 545.6 |
| | Standard error | 280.6 | 87.5 | 3.2 | 215.1 | 77.5 |
| | k parameter* | 0.66 | 0.71 | 0.72 | 0.71 | 0.68 |
| Males | Mean | 1602.8 | 410.8 | 13.6 | 851.6 | 326.8 |
| | Standard error | 154.3 | 61.8 | 2.8 | 111.9 | 39.2 |
| | k parameter* | 0.66 | 0.71 | 0.72 | 0.71 | 0.66 |
| Female/male ratio | | 1.7 | 1.3 | 1.3 | 2.0 | 1.7 |
| Total worms (females+males) | Mean | 4447.2 | 966.2 | 31.1 | 2577.5 | 872.4 |
| | Standard error | 431.1 | 148.2 | 5.6 | 323.0 | 115.3 |
| | k parameter* | 0.66 | 0.71 | 0.72 | 0.71 | 0.68 |
| Pearson correlation (P< 0.001) | | 0.619 | 0.915 | 0.728 | 0.501 | 0.404 |

^a Negative binomial parameter (P < 0.001).

The most prevalent species was *T. colubriformis*, followed by *H. contortus*, *T. circumcincta* and *O. venulosum*. The mean EPG in the 63 studied goats was 7660.8 (min 100, max 52,330). Combining the results of FEC and coproculture, *H. contortus* showed the highest egg output, followed by *T. colubriformis*, *T. circumcincta* and *O. venulosum*. The scatter plots of FEC and worm burden for each GI strongyle species are reported in Fig. 2. The Pearson correlation results showed a positive relationship between FEC and total GI strongyle worm burden ($r = 0.619$; $P = 0.000$). At species level, the highest positive relationship was found for *H. contortus* ($r = 0.915$; $P = 0.000$), followed by *O. venulosum* ($r = 0.728$; $P = 0.000$), *T. colubriformis* ($r = 0.501$; $P = 0.000$), and *T. circumcincta* ($r = 0.404$; $P = 0.000$).

4. DISCUSSION

This study demonstrated no evidence of a circadian rhythm in the FECs of GI strongyles in goats. The study did show, however, a significant relationship between FECs and adult parasite counts on consecutive days, especially for *H. contortus*. The lack of a significant effect of the hour of sample collection on FEC was also reported by Bennett (1990) for strongyle parasites of equines, whereas a circadian rhythm of egg excretion has been observed for other nematode species, e.g. *Heligmosomoides polygyrus* in wild wood mice (Brown et al., 1994) and *Passalurus ambiguus* in rabbits (Rinaldi et al., 2007a,c). The findings of the present study have important practical implications, since they demonstrated for the first time that faecal sampling for GI strongyle FEC can be performed at any moment of the day on a goat farm without affecting FEC values. These results can be likely extended to sheep and cattle farms, since all these ruminants often share the same parasitic genera and/or species. In the present study, as expected, GI strongyle FECs were affected by month of sampling, and this should be considered in the design of parasite control programs for goats in regions with similar climate and management (Veneziano et al., 2004; Cringoli et al., 2008). The FEC and worm burden results of the present study showed that most of the GI strongyle burden may occur in a small percentage of hosts. Indeed, the distribution of FEC (at any hour of sample collection) and adults (both females and males) was asymmetrically positive. These findings are in agreement with other studies on small ruminants (Barger, 1985; Cabaret et al., 1998; Hoste et al., 2001, 2002b).

Secondly, the Pearson correlation results of the present study showed a positive relationship between FEC and total GI strongyle worm burden ($r = 0.6$). This positive relationship was found for all the GI strongyle. A positive correlation between EPG and GI strongyle worm counts has been previously found in dairy goats from several temperate and steppe areas, in particular when *H. contortus*, the most prolific species, was present (Cabaret and Gasnier, 1994; Cabaret et al., 1998). In addition, Roberts and Swan (1982) also found a strong correlation between FEC and the total number of *H. contortus* adults in naturally infected sheep and, more recently, Beriajaya and Copeman (2006) demonstrated a strong relationship between FEC and worm burden in sheep experimentally infected by *T. colubriformis*.

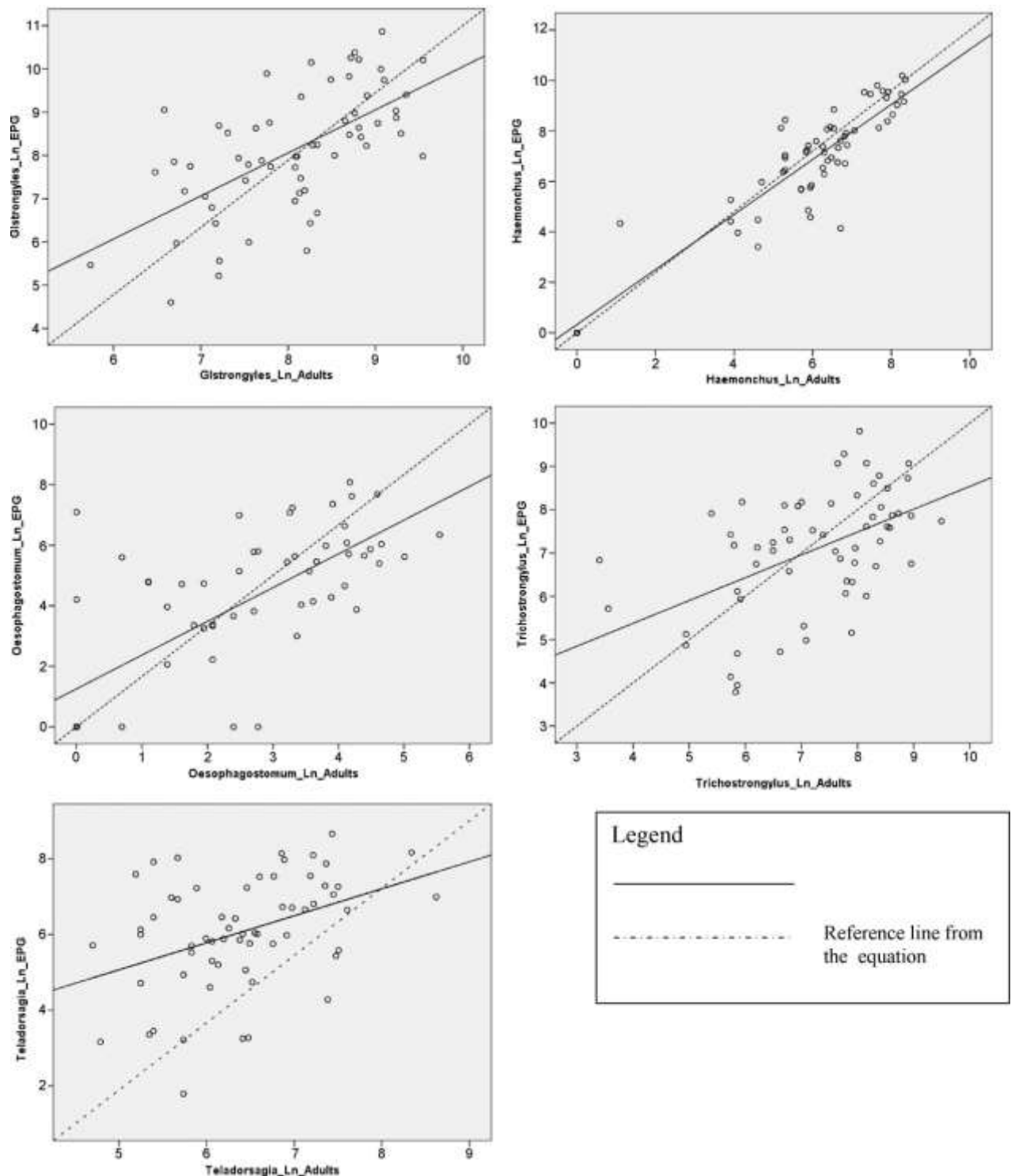


Fig. 2. Scattered plots of GI strongyle FEC and worm burden (ln-transformed data) for each GI strongyle species.

This relationship between FEC and worm burden may be influenced by many factors such as fecundity of species, age of worm, volume of ingesta and host resistance (Roberts and Swan, 1982). However, the association between FEC and

worm burden is not an illusion, even considering density-dependant phenomenon (Cabaret et al., 1998). The female–male ratio was similar to that observed in other studies (Coadwell and Ward, 1982; Paolini et al., 2003; Good et al., 2006). The strong linkage between FEC and worm burden supports the use of FEC techniques to measure the prevalence and intensity of infections for epidemiological surveys, to quantify the efficacy of treatments, and to detect anthelmintic resistance (Eysker and Ploeger, 2000). FEC also has potential in the identification of target animals for TST. To ensure cost effectiveness, however, further studies are needed to evaluate the costs of individual FEC on farms relative to other TST indicators. In conclusion, the present study showed that the hour of sample collection does not influence the GI strongyle FEC in goats and that there is a good relationship between FEC and total GI strongyle worm burden in goats. Gathering of this kind of information is recommended as an initial step for any host–parasite study (Villanua et al., 2006), and further research is needed on the influence of hour of sample collection and relationship with worm burden for other parasites of goats and for other ruminant species.

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CHAPTER 4

The maintenance of anthelmintic efficacy in sheep in a Mediterranean climate*

*Based on the manuscript: Rinaldi, L., Morgan, E.M., Bosco, A., Coles, G.C., Cringoli, G., 2014. The maintenance of anthelmintic efficacy in sheep in a Mediterranean climate. *Vet. Parasitol.* (Feb. 2014).

1. INTRODUCTION

Anthelmintic resistance has become an urgent global issue in the control of nematodes of sheep and goats in major small ruminant producing regions, e.g. South America, Australasia, South Africa and the UK, with multiple resistance found on many farms. This is particularly true where *Haemonchus contortus* is the dominant species in summer rainfall areas. There is relatively little information on what is happening in sheep flocks in countries with a Mediterranean type climate, i.e. hot dry summers and cooler moist winters, with the exception of Greece (Papadopoulos et al., 2001) where mixing of flocks on mountain pastures may slow the development of resistance, and Algeria where benzimidazole resistance was found on five out of 14 pilot farms and ivermectin resistance on one (Bentounsi et al., 2007). However, in south-western Australia which also has a Mediterranean type climate resistance has been developing very rapidly (Suter et al., 2005). The climate in central and southern Italy is typically Mediterranean and in central Italy resistance to imidazothiazole and macrocyclic lactones was found in trichostrongylids on a number of sheep farms (Traversa et al., 2007). In addition one case of benzimidazole resistance has been reported in *Trichostrongylus colubriformis* on a goat farm (Cringoli et al., 2007). In the southern regions of Italy sheep are kept for milk production with anthelmintic treatments usually being given only twice per year to lambs (Cringoli, personal communication, 2013) and adults being left untreated during lactation (Cringoli et al., 2008; Cringoli et al., 2009). Since these practices ought to leave abundant helminths *in refugia* for susceptible genotypes, and this is believed to be the most important issue in the development of anthelmintic resistance (Van Wyk, 2001), there should have been little development of resistance on farms using this system.

In the present study the presence of anthelmintic resistance was investigated on 54 farms using the faecal egg count reduction test (FECRT) following the recommendations of Coles et al. (1992) on flocks of sheep using four groups of anthelmintics (benzimidazoles, levamisole, ivermectin/moxidectin and monepantel) and the FLOTAC technique having a sensitivity of 2 eggs per gram of

faeces (Cringoli et al., 2010). The aim was to determine whether management practices in this region have allowed the maintenance of anthelmintic efficacy.

2. MATERIAL AND METHODS

2.1. Study area

The study was conducted in the Campania region of southern Italy. In this area, sheep farms are widely distributed with an average area of approximately 50 ha. The area is mainly used for cereal production but small pastures occur on upland areas that are unsuitable for cropping (Fig 1).



Fig. 1. Study area

2.2. Study farms and animals

Trials were conducted between 2008 and 2011 on 54 sheep farms.

Dairy sheep farms were randomly selected throughout the region and the selection was mainly driven by the availability of the farmer. The animals used for the trials were mainly local regional breeds, e.g. Bagnolese (for milk) and dairy crossbreeds (e.g. Comisana x Sarda) (Fig. 2). These animals were kept on the pasture all year round.



Fig. 2. Experimental animals

The anthelmintic classes, drugs and dose as well as the number of farms and animals used in the study are given in table 1. On each farm all animals were weighed and given the correct dose. With ivermectin a half dose was also included to indicate whether resistance to the macrocyclic lactones might be developing (Palmer et al., 2000). Tests were run with groups of sheep (12 to 20 animals per group) using six anthelmintics administered orally, levamisole (Levacide, Norbrook, 7.5 mg/kg) on 8 farms, ivermectin (Oramec, Merial, 0.1 and 0.2 mg/kg) on 8 farms, moxidectin (Cydectin, Pfizer, 0.2 mg/kg) on 3 farms, monepantel (Zolvix, Novartis, 2.5 mg/kg) on 8 farms, netobimin (Hapadex, Intervet, 7.5 mg/kg) on 22 farms (pooled samples) and albendazole (Sverminator/Valbazen, Fatro/Pfizer 3.8 mg/kg) on 5 farms (pooled samples). Pooled samples were used where the presence of benzimidazoles was being investigated so that more farms could be surveyed (Rinaldi et al., accepted). Faecal samples were collected rectally on days 0 and 7 for levamisole and monepantel and on days 0 and 14 for ivermectin and netobimin/albendazole.

Table 1. The anthelmintics (class, drugs and dosages) used on sheep farms (no. of farms, animals and presence of a control group) in southern Italy. Mean GI strongyle EPG and activity of netobimin, albendazole, levamisole, ivermectin, moxidectin and monepantel against GI nematodes calculated by the four methods (FECR1, FECR2, FECR3 and FECR4).

| Anthelmintics | | | Farm characteristics | | | Day 0 FEC (mean epg) | | Day 7 FEC (mean epg) | | Day 14 FEC (mean epg) | | The activities calculated by the different methods: min and max FECR (min and max lower confidence limits) | | | |
|---------------|-------------|------------------------|---------------------------|------------------------------|-----------------------------|-------------------------|---------------|-------------------------|---------------|--------------------------|---------------|------------------------------------------------------------------------------------------------------------|----------------------|----------|----------------------|
| Class | Molecule | Dosage of drug (mg/kg) | No. of sheep farms tested | No. of animals per treatment | Presence of a control group | Control group | Treated group | Control group | Treated group | Control group | Treated group | FECR1 | FECR2 | FECR3 | FECR4 |
| BZ/PBZ | Netobimin | 7.5 | 22 (April, 2008) | 20 (pooled) | No | - | 505.0 | - | - | - | 4.8 | - | - | 92.0-100 | 92.0-100 (85.9-99.9) |
| | Albendazole | 3.8 | 5 (March, 2008) | 20 (pooled) | No | - | 257.4 | - | - | - | 0 | - | - | 100 | 100 (94.4-98.3) |
| LV | Levamisole | 7.5 | 8 (August, 2009) | 12 | Yes | 425.4 | 372.7 | 353.6 | 1.9 | - | - | 98.3-100 (92.2-98.8) | 98.3-100 (92.0-99.3) | - | 98.5-100 (97.4-99.9) |
| ML | Ivermectin | 0.2 | 8 (March, 2009) | 12 | Yes | 218.0 | 302.4 | - | - | 148.5 | 0.6 | 99.5-100 (95.5-98.8) | 99.6-100 (97.0-99.6) | - | 99.7-100 (98.6-99.9) |
| | Ivermectin | 0.1 | 8 (June, 2009) | 12 | Yes | 218.0 | 283.2 | - | - | 148.5 | 0.3 | 98.8-100 (90.2-98.8) | 98.0-100 (90.4-99.6) | - | 96.4-100 (93.4-99.9) |
| | Moxidectin | 0.2 | 3 (October, 2010) | 20 | Yes | 618.3 | 550.6 | - | - | 306.3 | 0.2 | 99.9-100 (99.1-99.4) | 99.9-100 (99.5-99.6) | - | 99.9-100 (99.8-99.9) |
| AAD | Monepantel | 2.5 | 8 (August, 2011) | 12 | Yes | 425.4 | 509.3 | 353.6 | 2.4 | - | - | 97.3-100 (94.9-99.4) | 97.8-100 (95.7-98.5) | - | 97.0-100 (96.1-99.8) |

a BZ/PBZ = Benzimidazoles/Probenzimidazoles; LV=Imidazothiazoles/Tetrahydropyrimidines, ML =Macrocyclic lactones; AAD = Amino Acetonitrile Derivates

2.3. Laboratory procedures

In groups treated with LV (levamisole), ML (ivermectin and moxidectin) and AAD (monepantel), individual faecal egg counts were determined using the FLOTAC dual technique (Cringoli et al., 2010) with a sensitivity of 2 eggs per gram (EPG) of faeces, using a sodium chloride based flotation solution (FS2, specific gravity = 1.200). On the farms treated with BZ/PBZ (albendazole and netobimin) FEC on pooled samples were performed, namely, equal amounts from individual faecal samples were pooled in the laboratory into one sample before and after treatment for each farm (Rinaldi et al., accepted). In addition, on each sampling day a composite faecal culture was conducted for each group (MAFF, 1986). Third stage larvae were identified to the level of genus using the morphological keys proposed by van Wyk et al. (2004). When a coproculture had 100 or fewer third stage larvae, all were identified; when more than 100 larvae were present, only the first 100 examined were identified.

2.4. Statistics

On each faecal sampling occasion, arithmetic mean EPG was calculated as recommended by the WAAVP guidelines for evaluating the efficacy of anthelmintics in ruminants and, for each treatment group, percent efficacy (%) was calculated in terms of FECR on the different days (Coles et al., 1992; Dobson et al., 2009). Different formulae were used based on the presence of a control group and/or the use of pooled samples:

1) $FECR1 = 100 \times (1 - [T2/C2])$, where T2 represents the mean post-treatment FEC of the treated group, and C2 represents the mean post-treatment FEC of an untreated control group (Coles et al., 1992). Arithmetic means were used and 95% Confidence Intervals (CI) calculated using variance in treatment and control groups as set out in Coles et al. (1992).

2) $FECR2 = 100 \times (1 - [T2/T1][C1/C2])$, where T1 and T2 represent the mean pre- and post-treatment FEC of the treated group, respectively, and C1 and C2 represent the mean pre- and post-treatment FECs of the untreated control group, respectively (Dobson et al., 2009; Dash et al., 1988; Pook et al., 2002). Confidence intervals were estimated by bootstrapping. Thus, pre and post treatment individual FEC for T and C groups were simulated from the original values with

replacement, and FECR re-calculated. The procedure was repeated 10,000 times, and the 2.5 and 97.5 percentiles of the simulated results were used as 95% CI. The PopTools (CSIRO, Australia) add-in to Excel (Microsoft Corp, USA) was used for the simulations.

3) $FECR3 = 100 \times (1 - [T2/T1])$, where T1 and T2 represented the mean pre- and post-treatment FECs of the treated group (McKenna, 2006). This formula was calculated for farms without a control group and when pooled samples were used in the laboratory.

4) $FECR4 = 100 \times (1 - [T2/T1])$, where T1 and T2 represent the total number of eggs counted in all individuals before and after treatment, respectively (Dobson et al., 2012). This formula was calculated for farms on which individual or pooled FEC were conducted, whether or not control groups were included. Confidence intervals were estimated using the Jeffreys interval, as described in (Dobson et al., 2012). Thus, 95% lower confidence limit = $100 \times (1 - (BETAINV(0.975, T2+1, T1-T2+1)))$ and 95% upper confidence limit = $100 \times (BETAINV(0.025, T2+1, T1-T2+1))$, where BETINV is the cumulative inverse beta function. Microsoft Excel was used to estimate FECR and confidence intervals.

3. RESULTS

The genera of nematodes present (minimum and maximum percentages in each treatment group) at the time of treatment were: *Trichostrongylus* (68.9-80.4%); *Teladorsagia* (11.6-16.3%); *Oesophagostomum/Chabertia* (2.7-11.2%); *Haemonchus* (1.9-7.4%); *Cooperia* (0.6-2.6%) and *Bunostomum* (0-0.2%). There was no significant variation of this percentages in relation to year, whereas some variation were found in relation to the period of sampling, especially regarding *Teladorsagia* that showed the highest prevalence in autumn (unpublished data).

The efficacies of the anthelmintic treatments are given in table 1. Very high efficacy was obtained with all anthelmintics tested as follows (average FECR between farms): levamisole 99.3% (range 98-100%), ivermectin half dose 99.5% (98.0-100%), ivermectin full dose 99.9% (99.3-100%), moxidectin 100% (99.9-100%), monepantel 99.4% (97-100%), netobimin 99.1% (92-100%) and albendazole 100%. Lower confidence limits (LCL) were generally high and always above 95%

for monepantel and 99% for moxidectin. On two of the eight farms on which ivermectin was used, LCL when using a half dose was between 90 and 95% using one or more statistical methods; for the full dose, the minimum LCL was 95.5% using FECR1 and 97.8 using FECR2. For levamisole, two of the eight farms tested showed LCL below 95%, using one or both methods.

On one farm, a low efficacy of netobimin was observed (farm no. 9, FECR3 = 92%) and anthelmintic resistance was suspected. However, the farm was revisited after a period of 1 year, and the FECRT repeated at this time using a control group gave an efficacy of 100%. Faecal cultures suggested that there had not been a significant change in species composition between the two FECRT.

4. DISCUSSION

GI nematode infection (caused by different genera of nematodes, e.g. *Teladorsagia*, *Haemonchus*, *Trichostrongylus* and *Oesophagostomum*) remains one of the main constraints to small ruminant production in southern Italy (Musella et al., 2011; Dipineto et al., 2013) and so maintenance of anthelmintic efficacy is important to ensure high levels of production and animal welfare.

The present data suggest that anthelmintic resistance is rare in southern Italy and supports the idea that with correct management the development of resistance can be greatly reduced. However, it should be noted that one limitation of the present study is that some of the results date back more than 5 years old and were conducted between March and October. It is likely that the infection levels and the ratio inhibited/immature/adults differ between seasons but according to our diagnostic data the epidemiological scenario did not change significantly in the years of our study (2008-2011). However, a constant monitoring of the efficacy of anthelmintics in sheep in southern Italy is strongly recommended. It has been established that more frequent treatments select for anthelmintic resistance (Kenyon et al., 2009) so the use of about two anthelmintic treatments in sheep per year should reduce the rate at which anthelmintic resistance develops. Although albendazole, netobimin and moxidectin are licensed for use in dairy sheep, the withdrawal period and resultant loss of production of milk mean that in practice it is only administered during the dry period. Thus lactating ewes should provide an

important source of nematodes *in refugia* and *refugia* is believed to be very important in slowing development of resistance (van Wyk, 2001). An obvious method of spreading resistance is through the purchase of animals so the low movement of sheep between farms should help reduce the spread of resistance when it develops. Thus probably unintentionally the farming practices in southern Italy should slow the development of resistance. By contrast in western Australia treatment of sheep during drought probably encouraged the development of resistance as the major source of pasture contamination after the rains return will be worms surviving treatment (Geerts et al., 1997). Treatment of animals in southern Italy does not usually occur during or at the end of the drought thus avoiding this mechanism of selection for resistance. Studies performed in Greece (Papadopoulos et al., 2001) suggested that drought was an important issue in selecting for anthelmintic resistance in the Mediterranean zone. In southern Italy treatment is usually given in March and October when significant numbers of worm larvae are on pasture (Fig. 3).

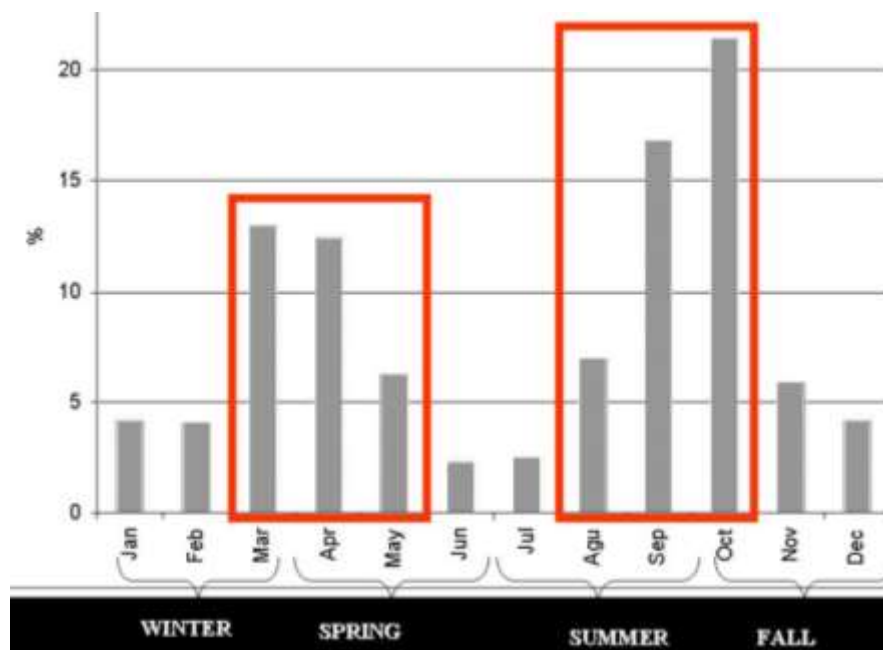


Fig. 3. Timing of anthelmintic treatments in sheep farms of southern Italy

Whilst the running of the FECRT may appear straightforward, one farm was of concern due to the relatively low efficacy when netobimin was used (92.0%). Repeat of the FECRT one year later showed an efficacy of 100% without change in

species composition. The reason for the initial lower value is not known but might be due to change in diet. This result indicates that a FECRT can sometimes underestimate the true efficacy and may suggest erroneously that low levels of resistance are present. Whilst this might not be important if used as the basis of advice to a farmer to change the type of anthelmintic used, since in that case loss of efficacy would be low in terms of production loss, it could lead to false positives in surveys of anthelmintic resistance and in scientific studies. Another possible limitation of the present study was that insufficient numbers of L3 larvae of *Cooperia* and *Chabertia* were found following faecal cultures to be sure that the high levels of efficacy found with the other genera would also be found with these. To assess possible macrocyclic lactone (ML) resistance on the farms, sheep were dosed both at the recommended dose rate (0.2 mg/kg) and at half this dose of ivermectin (0.1 mg/kg). This has been suggested as a simple method for the early detection of ivermectin resistance (Palmer et al., 2000). However, in the present study no suggestion of resistance was found using this method.

The present findings on the efficacy of anthelmintics against nematodes of sheep in southern Italy suggests that the percentage FECR for deciding whether anthelmintic resistant nematodes are present in sheep could be raised, particularly for ivermectin. This would have the advantage of permitting early detection of the beginning of anthelmintic resistance. To obtain more reliable data on possible low levels anthelmintic resistance a more sensitive egg counting procedure than the McMaster slide is required, especially when GI strongyle EPG values before treatment are low. Although FLOTAC devices were used in the present study these require centrifuging and thus the counts have to be lab based. With the introduction of Mini-FLOTAC (Cringoli et al., 2013) which is sensitive to 5 epg and can be used on farm, sensitive monitoring for anthelmintic resistance is no longer confined to the laboratory.

To reduce the cost of resistance testing for farmers the use of pooled faecal samples has been proposed (Morgan et al., 2005; Rinaldi et al., accepted) with equal weights of faeces taken from each sample with thorough mixing before counting. It has recently been confirmed that pooling is a valid method for the detection of anthelmintic resistance using the Mini-FLOTAC (Rinaldi et al., accepted). The pooling strategy was used in the present study to increase the

numbers of farms investigated for the possible presence of benzimidazoles resistance. There is a good correlation between averages of individual nematode egg counts and pooled samples in sheep (Nicholls and Obendorf, 1994; Morgan et al., 2005; Rinaldi et al., accepted). A disadvantage of pooled FEC for detection of anthelmintic resistance has been the inability to estimate confidence intervals when only a single figure for FECR is returned. A recently published, novel method for estimating such confidence limits (Dobson et al., 2012) could greatly improve the utility of pooled FEC in estimating anthelmintic efficacy. In the present study, results using this method agreed closely with those obtained using individual FEC, and it appears that it can provide a flexible and robust analysis for screening and confirmatory FECRT, especially when pooled FEC offer cost and logistical advantages.

The findings of the present study could be used when looking for the presence of low levels of anthelmintic resistance in ovine nematodes, especially when a sensitive egg counting procedure as FLOTAC is used. This would enable a small reduction in efficacy to be detected and this will be particularly valuable with anthelmintics which have a very high efficacy against nematodes of sheep with reduction in egg counts after treatment between 98 and 99%, as ML's (Cringoli et al., 2008, 2009) and monepantel (Hosking et al., 2009; Jones et al., 2010).

The possible changes that could be made to the definition of anthelmintic resistance in nematodes of sheep indicate that similar information should be prepared for all host species where anthelmintic resistance tests need to be undertaken, especially cattle, horses and other grazing animals.

In conclusion, the development of anthelmintic resistance on sheep and goat farms could be limited in countries with a Mediterranean type of climate provided that refugia of the nematode populations are maintained, anthelmintic use is restricted and movement of animals is not permitted to spread resistance.

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CHAPTER 5

Comparison of individual and pooled faecal samples in sheep for the assessment of gastro-intestinal strongyle infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC*

*Based on the manuscript: Rinaldi, L., Levecke, B., Bosco, A., Ianniello, D., Pepe, P., Charlier, J., Cringoli, G., Vercruysse, J. Comparison of individual and pooled faecal samples in sheep for the assessment of gastro-intestinal strongyle infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC. (Submitted to Vet. Parasitol., 2014)

1. INTRODUCTION

Infections by gastrointestinal (GI) strongyles continue to represent a serious challenge to the health, welfare and productivity of grazing ruminants throughout the world (Morgan et al., 2013). The specific and sensitive diagnosis of GI strongyle infections of livestock underpins effective disease control, which is now particularly important given the problems associated with anthelmintic resistance (AR) in parasite populations (Roeber et al., 2013a, b). Currently, diagnosis of these infections relies predominantly on copromicroscopy (Cringoli et al., 2010; Demeler et al., 2013) and faecal egg count (FEC) techniques are the most widely used methods to estimate GI strongyle intensity through the assessment of eggs per gram of faeces (EPG). Moreover, reduction in faecal egg count (FECR) is the method of choice to monitor anthelmintic drug efficacy and to detect AR in ruminants (Coles et al., 1992, 2006).

However, there are still some obvious limitations that will affect the use of FEC/FECR. From a general point of view, the main limitation of copromicroscopy is the time and cost to conduct FEC on a representative number of individual animals. An alternative to reduce the workload is to examine pooled (composite) faecal samples, in which equal amounts of faeces from several animals are mixed together and a single FEC is used as an index of group mean FEC. In their simulation-based study, Morgan et al. (2005) suggested that GI strongyle faecal egg density in a well-mixed composite sample from 10 sheep (3 g of faeces from each), estimated by examination of four McMaster chambers, is likely to provide an adequate estimate of group mean FEC in the majority of situations.

Similarly, examination of pooled samples in field studies was shown as a quick and valid alternative to the examination of individual samples for monitoring GI strongyle infections by means of FEC in sheep and cattle in Australia (Baldock et al., 1990; Ward et al., 1997). Some other studies have described the use of pooled FEC for assessing infections by helminths (not only GI strongyles) in sheep for farm routines and in cross-sectional prevalence surveys (Nicholls and Obendorf, 1994; Cringoli et al., 2002; Musella et al., 2011).

However, there are still many issues to be clarified before the pooled FEC is introduced in the routine diagnosis of GI strongyles and, by extension, in the assessment of anthelmintic drug efficacy (FECR) in ruminant farms.

First, the effect of pool size (i.e. the number of individual samples in each pool) has not been estimated so far and arbitrary numbers of individual faecal samples were used, ranging from 3 (Baldock et al., 1990) to 10 (Morgan et al., 1995).

Second, the effect of analytic sensitivity of the FEC technique on pooling has not been evaluated so far and the McMaster technique (Gordon and Whitlock, 1939; MAFF, 1986) was usually employed with an analytic sensitivity of 15 or 50 eggs per gram (EPG) of faeces. It is likely that a FEC technique with a higher analytic sensitivity might be used to pool a greater number of samples. The recently developed Mini-FLOTAC (Cringoli et al., 2013) (Fig. 1) having an analytic sensitivity of 10 EPG may provide an alternative to the commonly applied McMaster for quantitative copromicroscopy in ruminants (Da Silva et al., 2013) in order to perform FEC on pooled samples.

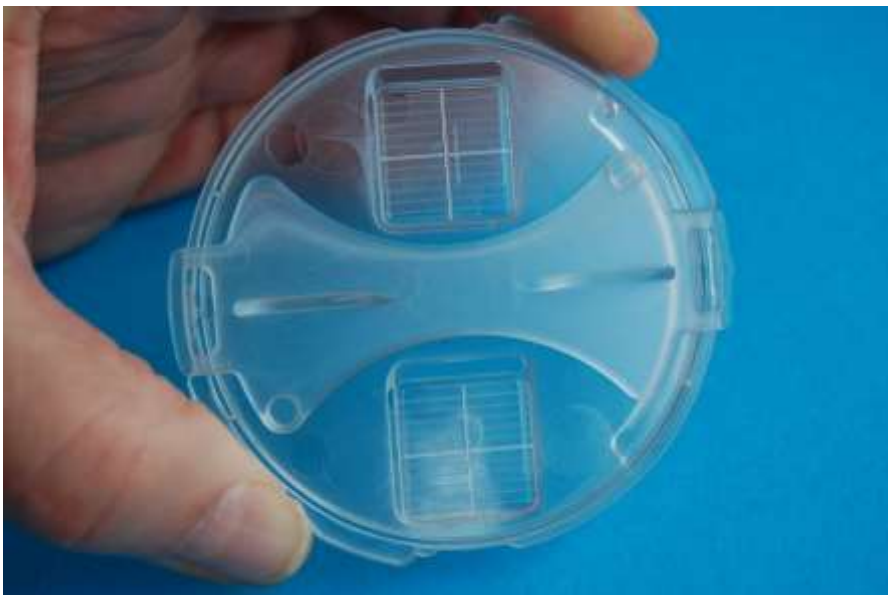


Fig. 1. Mini-FLOTAC

Third, there is a little information on the application of pooled FEC to decide on control programmes and in drug efficacy studies to assess FECR. In their recent

simulation study, Calvete and Uriarte (2013) report that pooling samples is one interesting option for FECR tests since it considerably reduces the workload.

In order to clarify some of these three key aspects concerning the effect of pooling faeces on FEC/FECR, the objectives of the present study were: (i) to further validate the pooling technique comparing FEC and FECR from individual sheep samples and pools of different size (5, 10 and 20 individual sheep samples), (ii) to assess the effect of three different analytic sensitivities (10, 15 and 50) on individual and pooled samples using McMaster (analytic sensitivities = 15 and 50) and Mini-FLOTAC (analytic sensitivity = 10); and (iii) to determine the effect of the pooling on FECR.

2. MATERIALS AND METHODS

2.1. Study design

Between October and December 2012, a study was conducted on 10 sheep farms located in the Campania region of southern Italy (Fig. 2). The animals on the farms were naturally infected with GI strongyles (*Trichostrongylus* spp., *Haemonchus contortus* and *Teladorsagia circumcincta*) (Dipineto et al., 2013). On each farm, individual faecal samples (at least 20 grams) from 20 adult sheep (when possible) were collected, before (D0) and after (D14) anthelmintic treatment with albendazole 3.75 mg/kg (Valbazen 19 mg/ml - oral suspension, Pfizer). For each farm and at each time point (D0 and D14) the 20 samples were numbered from 1 to 20.



Fig. 2. Experimental animals

All faecal samples were individually processed by the McMaster and the Mini-FLOTAC techniques as described below.

In addition, for each farm and at each time point (D0 and D14), the faecal samples were pooled in pools of 5 individual samples ($n = 4$), 10 individual samples ($n = 2$) and 20 individual samples ($n = 1$). All these pooled samples were prepared, using equal amounts from each individual faecal samples (2 grams) as shown in Figure 3.

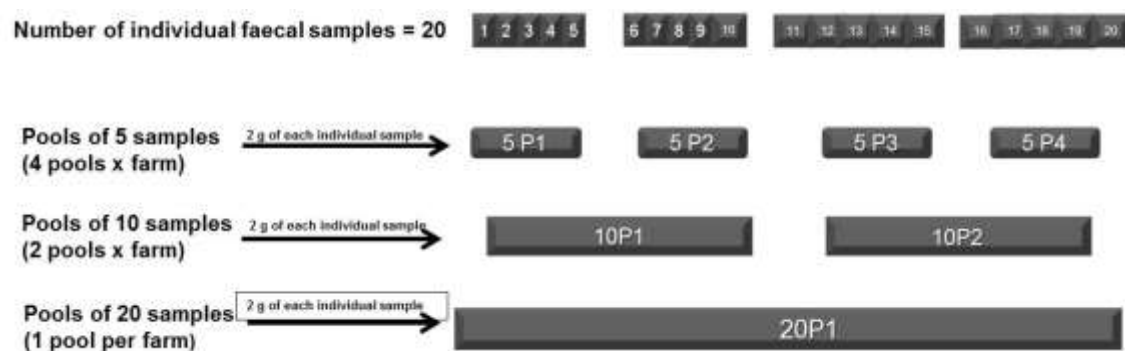


Fig. 3. Procedure to obtain pools of 5, 10 and 20 individual sheep faecal samples.

The total number of sheep farms and the total number of individual and pooled samples across the assessment of the infection intensity and the efficacy trial (D0 and D14) are provided in Figure 4. It should be noted that the predefined pool sizes of 5, 10 and 20 could not be met when <20 animals were sampled on a farm. Therefore, it was anticipated to have 80 pools of 5 (4 pools per farm x 10 farms x 2 occasions of sampling), 40 pools of 10 (2 pools per farm x 10 farms x 2 occasions of sampling) and 20 pools of 20 (2 pools per farm x 10 farms x 2 occasions of sampling) but the actual number of pools of different sizes is provided in Figure 2. However, it should be noted that in our analysis to verify differences in pool size, we considered all the samples met the predefined sample size. The pooled samples were stirred until homogenized. As for the individual samples, each pool was examined using McMaster and Mini-FLOTAC.

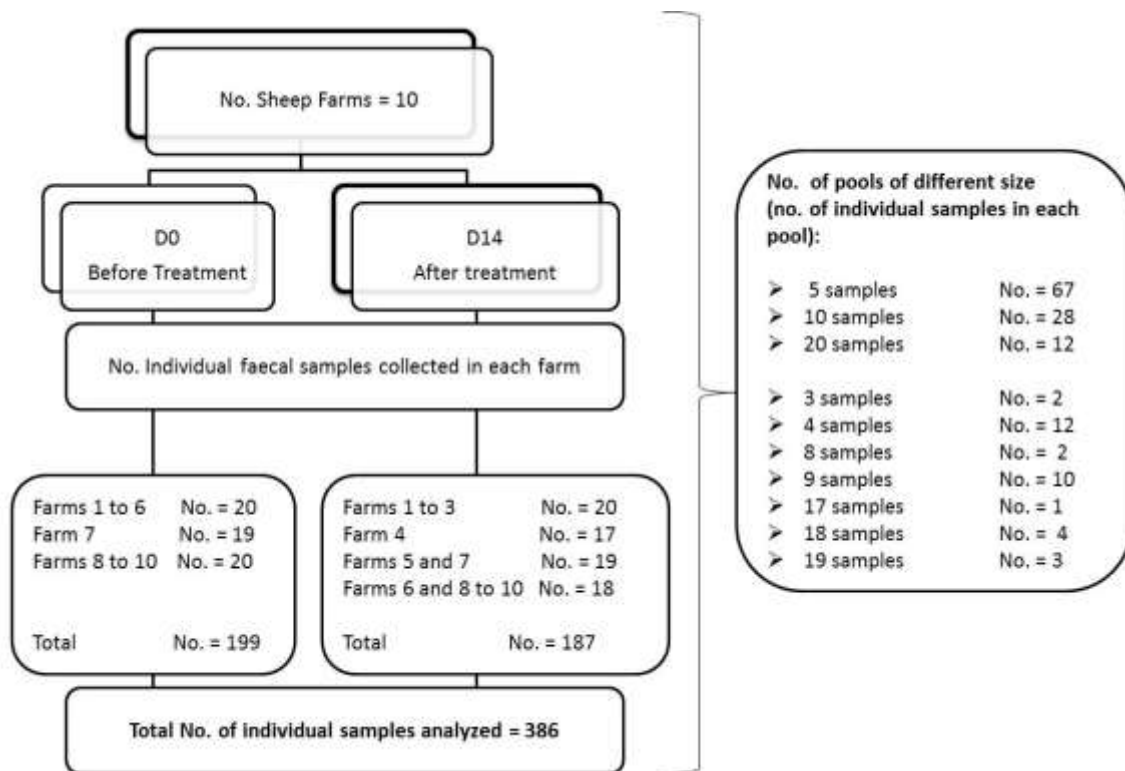


Fig. 4. Number of sheep farms, individual faecal samples and pools used for the study.

2.2. Parasitological examination

2.2.1. Modified McMaster technique

The modified McMaster technique (MAFF, 1986) was performed using the following standard operating procedure (SOP). Three grams of faeces were put into a container and 42 ml of sodium chloride (NaCl, specific gravity = 1.200) were added (dilution ratio = 1:15). The faecal suspension was thoroughly homogenized and strained three times through a wire mesh (aperture of 250 μ m) to remove large debris. The strained suspension was collected in a bowl and thoroughly mixed by pouring it 10 times in one bowl to another. Then, 0.5 ml aliquots were added to each of the two chambers of a McMaster slide (http://www.hawksley.co.uk/cell-count_glassware/05c_spec-chambers/). After 10 minutes, the GI strongyle egg counts were performed under the two grids (volume = 0.3 ml) and both chambers (volume = 1.0 ml) of the McMaster (Cringoli et al., 2004) under a light microscope using a 100x magnification. FEC values, expressed as EPG of GI strongyles, were obtained by multiplying the total number of eggs by 50 (McM50) or 15 (McM15).

2.2.2. Mini-FLOTAC technique

The Mini-FLOTAC technique (Cringoli et al., 2013) was performed using the following SOP. Two grams of fresh faeces were put into the Fill-FLOTAC container and 38 ml of sodium chloride (NaCl, specific gravity = 1.200) were added (dilution ratio = 1:20). The suspension was then thoroughly homogenized using the homogenizer stick of the Fill-FLOTAC. The faecal suspension was then filtered through the Fill-FLOTAC, and used to fill the two chambers of the Mini-FLOTAC. After 10 minutes, the top part of flotation chambers were translated and the Mini-FLOTAC was read under a light microscope using a 100x magnification. The analytic sensitivity of the Mini-FLOTAC basic technique was 10 EPG.

For both McMaster and Mini-FLOTAC, quality of the parasitological examination was ensured by (i) analyzing the samples within an average of 7 hours of collection, (ii) verification of density of the NaCl solution using a hydrometer, (iii) calibration of the scale weighing the faecal material, (iv) supervision of the pooling

procedures and (iv) reading the McMaster and Mini-FLOTAC by two senior researchers.

2.3. Statistical analysis

The statistical analyses were performed in the statistical software R (R Development Core Team, 2004). The level of significance was set at $p < 0.05$ for all tests.

2.4. Comparison of individual and pooled samples for assessment of FEC and drug efficacy (FECR)

The agreement in FEC between individual samples and pooled samples was verified by a permutation test (10,000 iterations) based on Pearson correlation coefficient and differences in EPG values for each of the pool sizes and FEC technique separately.

The anthelmintic drug efficacy at each farm was measured by means of FECR using the formula below:

$$FECR (\%) = 100\% \times \left(1 - \frac{\text{arithmetic mean of FEC at follow-up (D14)}}{\text{arithmetic mean of FEC at baseline (D0)}} \right)$$

As for FEC, the agreement in FECR between individual samples and pooled samples was verified by a permutation test (10,000 iterations) based on Pearson correlation coefficient and differences in FECR for each of the pool sizes and FEC technique separately. The Tukey's method was applied for multiple comparisons.

2.5. Comparison of diagnosis and assessment of drug efficacy across FEC techniques

Agreement in qualitative and quantitative diagnosis of GI strongyles

The three copromicroscopic techniques (Mini-FLOTAC, McM15 and McM50) were compared qualitatively (sensitivity) and quantitatively (FEC). Sensitivity was calculated for each technique, using the combined results of the techniques as the diagnostic 'gold' standard. Therefore, the specificity of both Mini-FLOTAC and McMaster was set at 100%, as indicated by the morphology of the eggs. Differences in sensitivity between techniques were assessed by a permutation test taking into

account the dependency of results within samples (10,000 iterations). The Tukey's method was applied for pair-wise comparison. The variation in sensitivity within each technique was explored by a logistic regression model, which was fitted for each of the techniques with their test result (positive/negative) as the outcome, and the mean FEC across techniques as covariate. The predictive power of this model was evaluated by the proportion of the observed outcome that was correctly predicted by the model. To this end, an individual probability >0.5 was set as a positive test result, and negative if different. Finally, the sensitivity for each of the observed values of FEC was estimated based on this model.

The agreement in FEC across the three techniques (Mini-FLOTAC, McM15 and McM50) was verified by a permutation test (10,000 iterations) based on Pearson correlation coefficient and differences in FEC. The Tukey's method was applied for multiple comparisons.

2.6. Agreement in assessment of anthelmintic drug efficacy (FECR)

We assessed the agreement across FEC techniques in classifying the drug efficacy into 'reduced' (= FECR $<95\%$ AND lower limit of the 95% confidence interval (LL of 95%CI) $<90\%$), 'suspected to be reduced' (= FECR $<95\%$ OR LL of 95%CI $<90\%$) and 'normal' (= FECR $\geq 95\%$ AND LL of 95%CI $\geq 90\%$) as described by Coles et al. (1992). The 95%CI was based on a nonparametric bootstrap (10,000 iterations). The agreement in classifying the drug efficacy was evaluated by a permutation test (10,000 iterations) based on the Kappa Fleiss statistic.

3. RESULTS

3.1. Comparison of individual and pooled samples for assessment of FEC and FECR

3.1.1. Agreement in assessment of FEC

The correlation between FEC results of pooled samples and mean of individual FEC is illustrated by Figure 5. Overall, FEC results of pooled samples correlated positively with the mean FEC of individual samples, with high correlation coefficients (R_s), i.e. ≥ 0.94 ($p < 0.0001$), regardless the pool size and the analytic sensitivity. Although these R_s values were high, the concordance plots illustrate a difference in level of agreement between the individual and pooled samples. This particularly for pool sizes of 10 and 20, for which FEC based on pooled samples result in lower estimates compared to FEC individual samples as FEC increase (FEC based on pooled samples are located below the line of equality, slope 1).

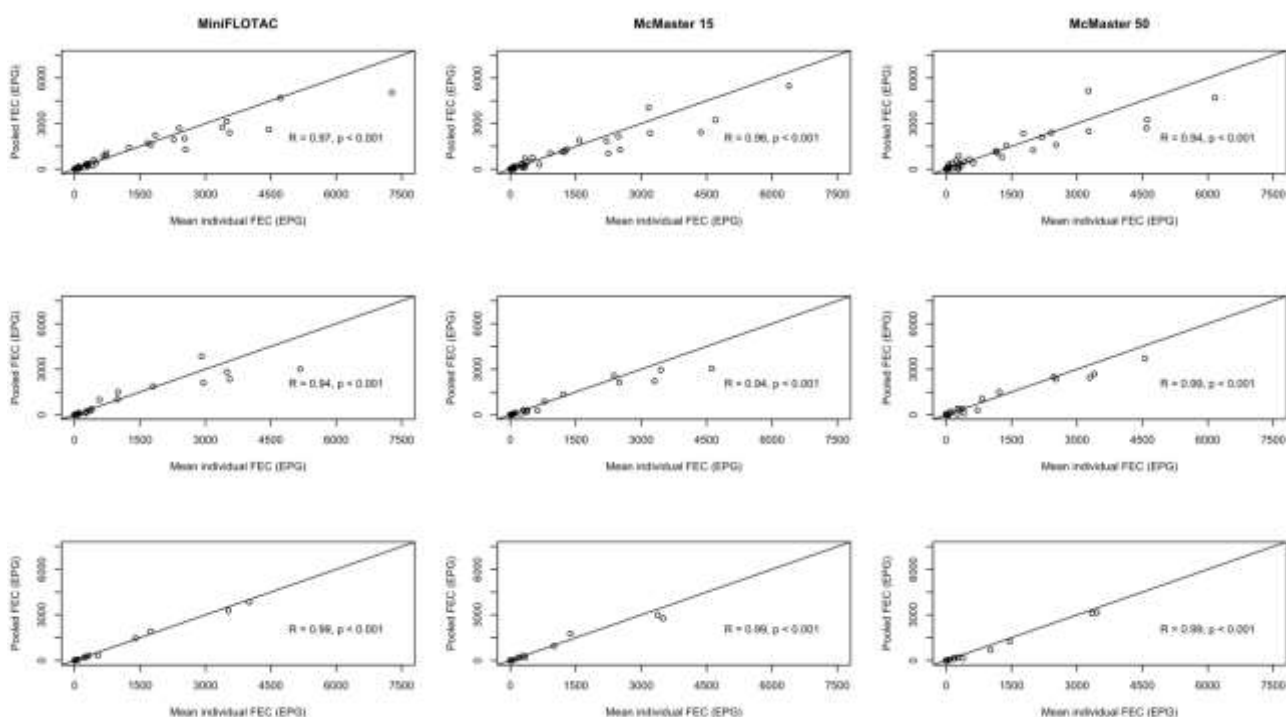


Fig. 5. The agreement in FEC based on the examination of individual and pools of 5 (top row), 10 (middle row) and 20 (bottom row) samples for three different copromicroscopici techniques. R: Pearson's correlation coefficient. The straight line represents the line of equality (slope = 1).

The difference in FEC between pooled and individual samples is summarized in Table 1. Overall, examination of individual samples resulted in higher FEC with differences in FEC ranging from 20 to 99 EPG. However, a significant difference in FEC was observed only for McM15 and when 10 samples were pooled. In this case, the mean difference between individual and pooled FEC was 99 ($p = 0.05$).

Table 1. The difference in FEC between examination of pooled and individual samples for Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

| Pair-wise comparison | Mean difference in FEC (EPG) | | |
|----------------------------|------------------------------|--------------|--------------|
| | <i>(p-value)</i> | | |
| | Mini-FLOTAC | McM15 | McM50 |
| Individual vs. pools of 5 | 90 (0.27) | 91 (0.10) | 56 (0.42) |
| Individual vs. pools of 10 | 86 (0.30) | 99 (0.05) | 68 (0.26) |
| Individual vs. pools of 20 | 20 (0.96) | 50 (0.68) | 76 (0.16) |

3.1.2. Agreement in assessment of FECR

Table 2 summarizes per farm the FECR for the different pool sizes for each of the three copromicroscopic techniques. With the exception of one farm (#4), pooling samples allowed for assessing FECR on all farms using all three FEC techniques. On this farm (#4), no FECR could be determined when using McM15 as the mean FEC of the pools post treatment were zero. This was also the case for pools of 10 and 20 when examined with McM50. With the exception of farms No. 2 and 3 (see Table 4), FECR was 100% when calculated for individual animals and across the different pool sizes ($n = 5, 10$ and 20 individual samples) and copromicroscopic technique (Mini-FLOTAC, McM15 and McM50). Given the low variation in FECR results, no

attempts were taken to verify correlation, and differences in FECR between the three methods. However, noteworthy on Farm 3 FECR (%) was constantly below 100% using Mini-FLOTAC when calculated for individual animals and across the different pool sizes ($n = 5, 10$ and 20 individual samples). Mini-FLOTAC actually found resistance at the pool size of $n=20$, whereas the other methods missed it.

3.2. Comparison of diagnosis and assessment of drug efficacy across FEC methods

3.2.1. Agreement in qualitative and quantitative diagnosis of GI strongyles

In 191 out of 386 (49.5%; 95% confidence intervals (95% CI) [44.4; 54.6]) samples GI strongyle eggs were detected with at least one of the three copromicroscopic techniques. Mini-FLOTAC allowed for the detection of eggs in all the 191 samples (sensitivity = 100%, 95%CI [100; 100]). The sensitivities of McM15 and McM50 were 88.5% [84.0; 93.0] and 75.9% [69.9; 82.0], respectively. Mini-FLOTAC was more sensitive compared to both McM15 and McM50 ($p < 0.001$). Furthermore, McM15 resulted in more sensitive test results compared to McM50 ($p < 0.001$). Figure 4 indicates that both McM15 and McM50 often fail to detect low FEC, and that this was more pronounced for McM50. However, both McM15 and McM50 became equally sensitive compared to Mini-FLOTAC when FEC increased. For both methods, the model could correctly predict the observed test results in more than 95% of the cases.

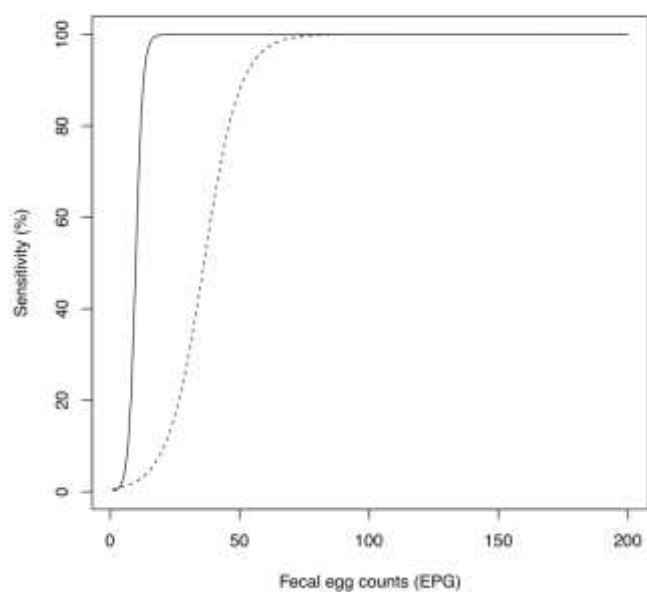


Fig. 6. The predicted sensitivity derived from logistic regression for McMaster based on the examination of the entire slide (McM15; straight line) and of the grids (McM50; dashed line). For both methods, the model could correctly predict the observed test results in more than 95% of the cases.

Table 2. The agreement in FECR across different pool sizes (N) and copromicroscopic techniques (Mini-FLOTAC, McM15 and McM50).

| Farm | Mini-FLOTAC | | | | McM15 | | | | McM50 | | | |
|---------|-------------|-------|-------|--------|-------|-------|-------|--------|-------|-------|-------|--------|
| | Ind | N = 5 | N =10 | N = 20 | Ind | N = 5 | N =10 | N = 20 | Ind | N = 5 | N =10 | N = 20 |
| | FECR | | (%) | | FECR | | (%) | | FECR | | (%) | |
| 1 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2 | 99.6 | 100 | 100 | 100 | 99.1 | 100 | 100 | 100 | 98.6 | 100 | 100 | 100 |
| 3 | 98.8 | 97.3 | 99.1 | 91.4 | 98.5 | 98.9 | 90.0 | 100 | 100 | 100 | 83.3 | 100 |
| 4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | – | 100 | 100 | – | – |
| 5 to 10 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Ind = individual samples

Table 3 summarizes the agreement in FEC across the three copromicroscopic techniques. There was a significant positive correlation for each of the three pair-wise comparisons (Pearson's correlation coefficient >0.95 , $p < 0.001$). However, Mini-FLOTAC resulted in significant higher FEC compared to both McM15 and McM50, with a mean difference in egg counts of approximately 90 EPG ($p < 0.001$). There was no significant difference in FEC across McMaster variants (mean difference of 3.9 EPG, $p = 0.97$).

Table 3. The agreement in FEC across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

| Pair-wise comparison | Pearson correlation coefficient (p -value) | Mean difference in FEC (p -value) |
|----------------------|-----------------------------------------------|--------------------------------------|
| Mini-FLOTAC vs McM15 | 0.98 (<0.001) | 90.9 (<0.001) |
| Mini-FLOTAC vs McM50 | 0.97 (<0.001) | 87.0 (<0.001) |
| McM15 vs McM50 | 0.99 (<0.001) | -3.9 (0.98) |

3.2.2. Agreement in assessment of anthelmintic drug efficacy (FECD)

Table 4 summarizes per farm the number of animals included in the efficacy trial, mean FEC at baseline, FECD and the final interpretation on drug efficacy for each of the three copromicroscopic techniques. At least 17 animals per farm were sampled both before and after the administration of the drug. There was a wide variation in mean FEC at baseline, ranging from 52 to 4078 EPG for Mini-FLOTAC, from 21 to 3599 EPG for McM15, and from 29 to 3539 EPG for McM50. This was in contrast with the drug efficacy results, for which FECD were higher than 98% and drug efficacy was assigned as having 'normal' drug efficacy on all farms, and this was independent of the copromicroscopic techniques. Given this low variation in FECD results and the lack of disagreement in the final interpretation no attempts were taken to verify correlation, and differences in FECD and the final interpretation between the three techniques.

Table 4. The agreement in FECR across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

| Farm ID | No. samples | Mini-FLOTAC | | McM15 | | McM50 | |
|---------|-------------|----------------|------------------|----------------|-----------------|----------------|-----------------|
| | | Mean FEC at D0 | FECR (95%CI) | Mean FEC at D0 | FECR(95%CI) | Mean FEC at D0 | FECR (95%CI) |
| 1 | 20 | 1396 | 100(99.9; 100) | 999 | 100(100; 100) | 1023 | 100(100; 100) |
| 2 | 20 | 261 | 99.6(98.7; 100) | 173 | 99.1(96.7; 100) | 175 | 98.6(94.4; 100) |
| 3 | 20 | 536 | 98.8(97.5; 99.5) | 341 | 98.5(96.4; 100) | 388 | 100(100; 100) |
| 4 | 17 | 52 | 100(100; 100) | 21 | 100(100; 100) | 29 | 100(100; 100) |
| 5 | 19 | 1830 | 100(100; 100) | 1444 | 100(100; 100) | 1529 | 100(100; 100) |
| 6 | 18 | 225 | 100(100; 100) | 219 | 100(100; 100) | 219 | 100(100; 100) |
| 7 | 18 | 4078 | 100(100; 100) | 3599 | 100(100; 100) | 3539 | 100(100; 100) |
| 8 | 18 | 3621 | 100(100; 100) | 3428 | 100(100; 100) | 3365 | 100(100; 100) |
| 9 | 18 | 360 | 100(100; 100) | 333 | 100(100; 100) | 314 | 100(100; 100) |
| 10 | 18 | 72 | 100(100; 100) | 54 | 100(100; 100) | 64 | 100(100; 100) |

4. DISCUSSION

The present study provided new insights towards standardizing FEC/FECR on pooled faecal samples in sheep for the assessment of GI strongyle infection intensity and anthelmintic drug efficacy. In particular, the effect of different pool sizes and analytic sensitivities on pooled FEC/FECR was evaluated.

Significant findings emerged regarding: (i) agreement between individual samples and pooled samples in assessment of FEC using the different analytic sensitivities (10 EPG using Mini-FLOTAC, 15 and 50 EPG using McMaster); (ii) agreement between individual samples and pooled samples in assessment of anthelmintic drug efficacy (FECR) using different analytic sensitivities; and (iii) qualitative (sensitivity) and quantitative (FEC) performance of the FEC methods.

First, regarding the agreement between individual samples and pooled samples in assessment of FEC, our findings showed that GI strongyle EPG of pooled samples correlated positively with mean EPG of individual samples, with high correlation coefficients (≥ 0.94) regardless pool sizes and analytic sensitivities. Despite this high correlation, there was an apparent, but insignificant underestimation of FEC when samples are pooled, which may need further attention. Nevertheless, in line with previous studies our findings support the potency of pooling strategy to reduce the workload in the laboratory. However, it is important to note that this study was not designed to verify to which extent the outcome of one pool of 5, 10 or 20 individual samples represents the average infection intensity at the flock level. Although, this would clearly further decrease the workload in both the field (fewer animals needed to be sampled) and the laboratory (only one FEC), this approach, as illustrated by Morgan et al. (2005), may resulted in a thwarted interpretation.

Second, concerning drug efficacy, with the exception of two farms, the present study showed FECR values of 100% when calculated for individual animals and across the 3 different pool sizes and analytic sensitivities. Therefore, as for FEC, the pooling approach worked very well also for FECR regardless of whether the pool was made up of 5, 10 or 20 individual samples, supporting previous studies. The very high drug efficacy found in the present study confirms that AR is rare in sheep of southern Italy, a region with a Mediterranean type of climate where the management system guarantees the maintenance of nematode populations *in*

refugia, and anthelmintic use is limited (Cringoli et al., 2008, 2009; Rinaldi et al., accepted). However, the main limitation of these findings on FECR is represented by the high efficacy (100% in most of farms) of anthelmintics found in the present study. Therefore, further studies are required to assess the validity of FECR on pooled faecal samples also in settings where the efficacy of anthelmintics is less than 95% and AR is suspected.

Third, regarding the sensitivity of the FEC techniques, as expected, our findings showed that Mini-FLOTAC was more sensitive compared to the two variants of McMaster (McM15 and McM50) for the diagnosis of GI strongyles in sheep (100% vs 88.5% vs 75.9%). Both McM15 and McM50 often failed to detect low GI strongyle EPG but became equally sensitive compared to Mini-FLOTAC when FEC increased, thus confirming the findings of other studies on comparison of FEC techniques (e.g. Rinaldi et al., 2011; Levecke et al., 2011, 2012a,b). Mini-FLOTAC also resulted in significant higher FEC compared to both McMaster variants, with a mean difference in egg counts of approximately 90 EPG ($p < 0.001$). However, it remains unclear to which extent this difference has a biological and/or practical impact. We still do not know what is the EPG threshold above which it is advisable to intervene with a specific control program, for example using a targeted treatment or a targeted selective treatment approach. All these questions and considerations underline that it is imperative to pay more attention to the final interpretation of FEC prior to recommend any FEC technique and any analytic sensitivity.

In addition, there is a lack of information regarding the actual cost-effectiveness of the pooled approach in copromicroscopy. It would be therefore advisable to conduct a comparative cost assessment study of individual and pooled FEC/FECR taking also in consideration the effect of different pool sizes and analytic sensitivities (e.g. McMaster *versus* Mini-FLOTAC). Valid examples of reliable and precise methodologies for assessing cost-effectiveness in copromicroscopy can be taken from the literature (e.g. Levecke et al., 2009; Speich et al., 2010).

Overall, the results of our study showed that pooling faecal samples can be used for FEC and FECR. Our findings are in line with recent studies on the same topic. As an example, pooled FEC was successfully used in horses (Eysker et al., 2008) for monitoring helminth control. Furthermore, Daniel et al. (2012) used FECR on pooled samples to assess the efficacy of triclabendazole against *Fasciola hepatica* in sheep farms in the UK. Concerning public health, Mekonnen et al. (2013)

highlighted that pooling stool samples is a promising approach for rapidly assessing infection intensity of soil transmitted helminths in humans as well as for drug efficacy studies. Finally, in their recent computer-based simulation study, Calvete and Uriarte (2013) suggest that the diagnostic performance of the FECR test (also using a pooled approach) should be re-evaluated and the recommendations of the W.A.A.V.P. should be updated as already reported in Levecke et al. (2012a).

In conclusion, the present study highlighted that pooling ovine faecal samples is a rapid procedure that holds promise as a cost-effective, but at the same time accurate strategy for assessing the intensity of GI strongyles (FEC) in sheep as well as anthelmintic efficacy (FECR). However, further research is required (i) to determine biological and epidemiological significance of FEC in sheep farms in order to establish the EPG thresholds for control programs; (ii) to verify in-depth the cost-effectiveness of pooled FEC compared to individual FEC; (iii) to optimize and standardize the methodology of pooling faecal samples; (iv) to verify the validity of the pooled FECR test also in settings where anthelmintic efficacy is less than 95%; and (v) to assess the performance of pooling FEC for copromicroscopic diagnosis of helminths other than GI strongyles and protozoa in sheep as well as in other livestock species.

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CHAPTER 6

Overall Discussion

1. THE STRATEGY OF MONITORING INFECTIONS BY GI NEMATODES IN SMALL RUMINANTS

As described in the previous chapters, faecal helminth egg counting has become a routine part of small ruminant veterinary practice, creating a context for interaction with farmer clients that is a prerequisite for effective health planning. Sheep helminth egg counts are performed in the investigation of failure of lambs to reach production targets or as ancillary diagnostic tests for the clinical examination of ill-thrifty, scouring or anaemic animals. Helminth egg counts are also used to monitor parasite control programmes, including the management of anthelmintic resistance. The most appropriate egg counting method, the numbers of samples required and interpretation of the results depend on the nature of the problem being investigated (Demeler et al., 2012). However, it should be noted that ancillary diagnostic tests, such as FEC are of limited value without the context of a full veterinary clinical examination. The diagnosis of helminth parasitism must involve a relevant history, placing the problem in context and focussing on previous grazing management and anthelmintic drug treatments in particular. The number of animals affected with scour, anaemia and subcutaneous oedema needs to be noted, along with the extent and severity of the clinical signs. Post-mortem examination of freshly dead carcasses can be useful, whenever the opportunity arises, enabling the identification of the nematode parasites, assessment of associated pathology and total worm counts to be performed.

There has been a tendency towards rigid interpretation of the results of FEC, propagated by diagnostic laboratories' perceived need to interpret their reports without knowledge of the situation to which they pertain, and by a general veterinary wish for prescriptive information, whereby reference ranges have been created to indicate absence of, moderate or subclinical, significant and severe parasitism. However, such rigid interpretation is generally unhelpful with regards to assessment of the helminth burden or need for treatment. Egg counts provide valid information about the presence of patent helminth infections, but the value of information concerning numbers of nematode eggs per gram (EPG) of faeces depends on the manner in which it is interpreted.

Egg counts are presented on the basis of the ratio of EPG, hence their interpretation depends on knowledge of the relative faecal dry matter content, feed intake and of the manner in which the animals were fed at the time of sampling. The faecal dry matter may be influenced by host responses to nematode parasitism (Colditz, 2008), altering the ratio of EPG, but with no overall effect on the total number of eggs shed. Variation in egg production in relation to the numbers and pathogenicity of adult female nematodes needs to be accounted for, hence an understanding of these relationships, consideration of temporal regulatory influences on egg production of female nematodes (Stear et al., 1995) and knowledge of the helminth genera that are present is required. The eggs of some genera are characteristic, while those of *Teladorsagia*, *Haemonchus*, *Trichostrongylus* and *Cooperia* are not easily distinguishable without resorting to morphometrics, fluorescent agglutinin staining, coprocultured larval identification, or molecular methods (Roeber et al., 2013). Hence, in the absence of these further tests, it is necessary first to extrapolate which trichostrongyle genera are likely to be present, before interpreting the egg counts. It is also important to evaluate any effects of sheep breed, age, reproductive status and management on faecal helminth egg counts.

1.1. Why monitoring infections with GI nematodes in small ruminants

The principal reason for farming sheep is to convert primary forage, herbage or cereal crops into a marketable product, hence the profitability of global sheep production is heavily influenced by the efficiency of conversion of these primary crops into meat, wool, milk, or skins. Indeed, infections of the gastrointestinal nematodes (GIN) affect not only the quantity of sheep milk produced but also the quality as reported in Scala et al (2012). The feed to meat conversion efficiency is greater in sheep that achieve maximal growth rates than in ill thrifty animals, because there is a daily nutritional requirement for maintenance which must be met before growth can occur, irrespective of the time taken to reach slaughter weight. Furthermore, sheep which are slow to finish are more susceptible to compounding effects of production limiting diseases such as helminth parasitism, trace element deficiencies and respiratory disease than rapidly growing animals which may leave the farm before the main risk period for these problems. It is also

intuitive that net greenhouse gas emissions from efficiently produced ruminant livestock are less than those from ill thrifty animals, reflecting differences in the amounts of feed that must be metabolised per kg of meat produced. World-wide, helminth parasites are arguably the most important causes of suboptimal productivity in sheep, hence their sustainable control is a prerequisite for economically efficient farming, in particular with GIN continue to represent a serious challenge to the health, welfare and productivity of grazing ruminants throughout the world (Morgan et al., 2013). All grazing animals are exposed to helminth infections at pasture and any respective future intensification of livestock farming will increase the risk of helminth disease. The ranking of GIN as one of the top cause of lost productivity in small and large ruminants by the recent DISCONTTOOLS programme (<http://www.discontools.eu/home/index>) witnesses the increasing EU's consideration of the impact of these parasites upon animal health, welfare and productivity. In addition, Scala et al. (2011) demonstrated the negative effects of GIN on the fertility factor of sheep. The economic costs of parasitic disease are currently difficult to quantify, however some estimates do exist within the scientific literature. Within the EU as a whole, annual sales of anthelmintic drugs used to control these infections in ruminants have been estimated to be in the order of 400 million € (Selzer, 2009). It is likely that these figures only represent the tip of the iceberg when it comes to calculating the true cost of livestock helminthoses endemic within the EU (Charlier et al., 2009).

2. THE NEED OF COPROLOGICAL EXAMINATIONS TO CONTROL INFECTIONS WITH GI NEMATODES IN SMALL RUMINANTS

As mentioned above and the previous chapters, FEC is of primary importance towards monitoring and controlling GIN infections in small ruminants. In particular , the results of FEC are important in order to: estimate infection intensity (McKenna, 1987; McKenna and Simpson, 1987), determine the degree of contamination with helminth eggs (Gordon, 1967), assess the effectiveness of anthelmintics (Waller et al., 1989), determine the breeding value of an animal when selecting for worm resistance (Woolaston, 1992), and guide control and treatment decisions (Brightling, 1988).

FECRT is currently the only test that can be used to detect resistance to all nematode species and anthelmintics in all hosts (McKenna, 2013). FECRT guidelines are made available by the World Association for the Advancement of Veterinary Parasitology (WAAVP). These guidelines (Coles et al., 1992) provide recommendations on the experimental set up (randomized control trial), sample size (≥ 10 or ≥ 15 animals per treatment group, each excreting at least 150 EPG), the FEC method (McMaster), statistical analysis (FECRT based on the arithmetic mean of grouped FEC after drug administration) and criteria defining reduced efficacy (FECRT $< 90\%$ or FECRT $< 95\%$ and lower limit of 95% confidence interval $< 90\%$). Several host-parasite-related factors could strongly affect FEC and therefore these factors should be taken into consideration when interpreting FEC results. Therefore, FEC alone should not be used to make a diagnosis or guide treatment decisions, but should be interpreted in conjunction with information about the nutritional status, age and management of sheep/goats in a flock (McKenna, 2002).

3. WHY COPROLOGICAL EXAMINATIONS CAN BE USED E.G. TO DECIDE THE NEED FOR CONTROL, TO DETERMINE EFFICACY OF TREATMENTS AND TO MONITOR CONTROL PROGRAMMES

Various approaches are used for the control of gastrointestinal helminths of livestock. These are based on three basic principles (Hoste and Torres-Acosta, 2011):

- i. The first principle is to reduce the exposure of the host to the infective stages (L3s), mainly achieved by strategies of grazing management (e.g. rotational grazing, alternate grazing, pasture-spelling and/or reduced stocking rates).
- ii. The second principle targets the development of a more favourable response of the host to gastrointestinal parasite infection (e.g. achieved through vaccination, improved nutrition, genetic selection of hosts and breeding for resistance).
- iii. The third principle directly targets the elimination of worms from their hosts through the administration of conventional (synthetic formulations) or non-conventional (plant or mineral) anthelmintic compounds.

Following the introduction of phenothiazines in the 1950's, the control of gastrointestinal

parasites has been achieved using chemical anthelmintics (Hoste and Torres-Acosta, 2011) and still predominantly relies on the treatment with broad-spectrum parasiticides.

The frequent and often uncontrolled use of these drugs has led to widespread problems with AR in parasites of livestock (Taylor et al., 2009). AR in parasites of veterinary importance has emerged as a major bionomic and economic problem worldwide, being currently most severe in parasitic nematodes of small ruminants (von Samson-Himmelstjerna, 2006; Waller, 1994, 1997).

Although there is hope for new, effective anthelmintics, there is also a major need to preserve those that we currently have at our disposal. Hence, monitoring the drug-susceptibility and -resistance status of strongylid nematode populations in livestock must be a high priority and should be an important component of integrated management strategies.

To date, genetic resistance against parasites is considered to be linked with the MHC and IgE genes. Furthermore, several gene detection studies based on the genome scan approach for this trait are currently being carried out on both crossed experimental populations (fat x lean Blackface lines and Sarda x Lacaune) and pure breeds (Churra) (Carta and Scala, 2004).

The major method for estimating levels of drug-susceptibility/resistance in strongylid nematodes of small ruminants remains the faecal egg count reduction test (FECRT) that can be used with all anthelmintic groups (Coles et al., 1992). Nematode eggs are counted in faeces at the time of treatment and at defined times after treatment, the time depending on the anthelmintic group used. A controlled efficacy test is the most reliable method of confirming anthelmintic resistance but expense usually excludes its use. Nevertheless, it is the gold standard for detecting anthelmintic resistance. Guidelines evaluating the efficacy of anthelmintics have been published elsewhere and should be adopted for investigating anthelmintic resistance (Wood et al., 1995; Duncan et al., 2002).

4. HOW CAN WE PROMOTE THE USE OF FLOTAC, MINI-FLOTAC AND OTHER COPROLOGICAL TECHNIQUES IN ITALY

Proper FEC techniques are imperative for the accurate diagnosis of GIN in small ruminant.

It is widely accepted that to be useful, diagnostic methods must be accurate, simple and affordable. They must also provide a result in time to institute effective control measures, particularly treatment (Banoo et al., 2010). ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable) diagnostic tests are needed (Banoo et al., 2010). From a general point of view, the method of copromicroscopy to be chosen will also depend on what the information is going to be used for. Also the use of highly sensitive techniques, such as the FLOTAC and Mini-FLOTAC techniques, is of primary importance to obtain more reliable data on possible low levels (EPG) of GIN and to detection of a beginning of anthelmintic resistance. Although FLOTAC devices were used in several studies of this PhD, it should be noted that they require centrifuging and thus the counts have to be lab based. With the introduction of Mini-FLOTAC (Cringoli et al., 2013) which is sensitive to 5 EPG and can be used on farm, sensitive monitoring for anthelmintic resistance is no longer confined to the laboratory (Rinaldi and Cringoli, 2014).

To reduce the cost of resistance testing for farmers the use of pooled faecal samples has been proposed (Morgan et al., 2005; Rinaldi et al., 2014) with equal weights of faeces taken from each sample with thorough mixing before counting. The pooling strategy was used in the present study to increase the numbers of farms investigated for the possible presence of benzimidazoles resistance. In the study described in the Chapter 5 of this thesis, results using this method agreed closely with those obtained using individual FEC, and it appears that it can provide a flexible and robust analysis for screening and confirmatory FECRT using the Mini-FLOTAC technique, especially when pooled FEC offer cost and logistical advantages.

5. STRATEGY OF SAMPLING, RECOMMENDATIONS

The global problem of anthelmintic resistance in small ruminants ensures that attention also needs to be given to the sustainability of anthelmintic treatment regimes as well as to their immediate economic benefit (Cringoli et al., 2007a,b, 2008). Different pathophysiological and performance indicators of GI strongyle infection in small ruminants have been recently proposed; for example, the FAMACHA system (van Wyk and Bath, 2002), body condition scoring (van Wyk and Bath, 2002) and liveweight gain and milk production (Hoste et al., 2002a,c) are also potential methods for identifying animals requiring anthelmintic treatments. However, the value of these methods in different climates and the benefits to animal productivity need further research (Ketzis et al., 2006) and faecal egg count (FEC) techniques remain the most common approaches for the estimation of prevalence and intensity of GI strongyle infections (Cringoli et al., 2004; Villanua et al., 2006).

FEC results may be affected by many factors, either depending on FEC techniques or depending on biological factors or sampling mode. The number of animals to test and frequency of sampling for the FEC performed on faecal samples taken from single animals are seldom informative (Sargison, 2013). In small ruminants, GIN egg counts are generally performed on samples taken from 10/20 animals within a group, and usually show standard deviations that are similar to the arithmetic mean values. Veterinarians, parasitologists and their staff should re-evaluate their attitude of “it’s only a faecal sample” and should therefore consider that suitable and timely sampling is the pre-requisite for interpreting the results of FEC in order to: estimate infection intensity (McKenna, 1987; McKenna and Simpson, 1987), determine the degree of contamination with helminth eggs (Gordon, 1967), assess the effectiveness of anthelmintics (Waller et al., 1989), determine the breeding value of an animal when selecting for worm resistance (Woolaston, 1992), and guide control and treatment decisions (Brightling, 1988).

Noteworthy, the findings of the present PhD demonstrated that faecal sampling for GI strongyle FEC can be performed at any moment of the day in goat farms without affecting FEC values. This notion can be likely extended to sheep and cattle farms, since all these ruminants often share the same parasitic genera and/or species (Rinaldi et al, 2009).

6. FUTURE OF COPROMICROSCOPY IN SMALL RUMINANTS

Although widely used for diagnosis of intestinal parasites, it is well known that copromicroscopy is prone to a number of shortcomings (Utzinger et al., 2012).

First, there is a clear lack of standardization of copromicroscopic techniques and usually each lab uses “its own” method mostly based on the “lab traditions” rather than on the performance or operational characteristics of the technique. However, it is important to underline that different factors may influence the performance of any copromicroscopic technique, especially those based on flotation (e.g. McMaster, Wisconsin, FLOTAC and Mini-FLOTAC) and sedimentation.

Second, the results of any copromicroscopic technique strongly depend on the accuracy of laboratory procedures but also on the experience of the laboratory technicians reading the microscopic fields (Becker et al., 2013). Hence, the reliable identification of parasitic infections requires in-depth training for specimen preparation, and expertise and experience for subsequent microscopic examination (Utzinger et al., 2012).

Third and most importantly, the main limitation of copromicroscopy is the time and cost to conduct copromicroscopic analysis (in particular FEC) on a representative number of individuals. However, this limitation can be overcome by performing FEC on pooled samples, in which equal amounts of faeces from several individuals are mixed together and a single FEC is used as an index of group mean FEC (Morgan et al., 2005). Recently, such pooling approaches have been applied to sheep faecal samples using Mini-FLOTAC and that has been demonstrated as a rapid procedure that holds promise as a valid strategy for assessing GIN infections in ruminants as described in Chapter 5.

Noteworthy, together with pooling, one of the challenges of the future of copromicroscopy is to perform diagnosis of neglected parasitic infections directly in the field by using field portable kits including the new generation of field microscopes. This aspect has had a rather long track record of development with a prior microscope models. As an example, Stothard et al. (2005) reported a field evaluation of a handheld microscope for diagnosis of intestinal schistosomiasis in Ugandan school children. This handheld microscope was suggested as a pragmatic alternative to the compound microscope, playing an important role in the

collection of prevalence data to better guide anthelmintic drug delivery and also empower the diagnostic capacity of peripheral health centers where compound microscopes are few or absent (Stothard et al., 2005). More recently, Bogoch et al. (2013) described the proof of concept that a mobile phone can be converted into a microscope for the point-of-care diagnosis of STHs in resource-constrained settings. It is therefore evident that using portable field microscopes without the need of electricity would be the optimal solution for the diagnosis of helminths in health periphery (for STH) and on farms (for GIN). A closed diagnostic device as Mini-FLOTAC could be easily “attachable” to such kind of microscopes supplied with adaptors for mobile phone camera (Fig.1).



Mini-FLOTAC under a compact portable microscope with a mobile phone.



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